

STUDIES ON HUMAN T LYMPHOCYTE COLONIES

by

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S U M M A R Y

This thesis examined the ability of human T lymphocytes to form colonies in semisolid agar.

A two step procedure requiring a continued supply of PHA, a source of SRBC during the agar culture and a high agar concentration to restrict the freely mobile proliferating cells was developed for the culturing of human lymphocytes in semisolid agar. The technique was later modified by replacing PHA with an IL2-containing lymphokine during the agar phase. The T cell nature of the colonies was confirmed by their ability to form E-rosettes. An absence of colonies from E-rosette depleted cells compared to E-rosette enriched cells strengthened this finding and indicated that the colony forming cells were T cells. The requirement for SRBC was investigated and it was found that red cells from a variety of mammalian sources supported colony formation through the elaboration of soluble factors which diffused through the agar and did not require cell-cell contact for their expression.*

Experiments relating to the cellular interactions involved in colony formation emphasised that cooperation between at least three cell populations was essential. The interaction of these cells could be described by two reactions. The first reaction required an adherent cell (probably monocyte) population which, together with PHA, provided the necessary signals for T cell activation. The activated T cells interacted during the second or proliferative reaction. In response to PHA, accessory T cells released IL2 which permitted colony forming cells to proliferate. Thus PHA-induced T colony formation required the cooperation of adherent cells for the activation reaction ($T \rightarrow T'$)

* See page xiii for abbreviations.

and IL2-producing T cells for the proliferative reaction ($T' \rightarrow nT'$). The microfilament/microtubule network and the redistribution of receptor:membrane complexes appeared to be an important feature of T cell function with regard to both mitogen and IL2 binding.


Utilising the colony forming ability of T lymphocytes studies on radiation induced mitotic death were carried out. Colony formation was sensitive to low doses of irradiation although PHA offered a limited degree of protection when cells were irradiated prior to, or in the early stages of activation. This protection could be explained by activities of repair enzymes which coincided with early cellular activation events.

The human T lymphocyte colony assay was found to be a valuable in vitro model for studying the steps involved in T lymphocyte activation and proliferation.

STATEMENT REGARDING ORIGINALITY

The investigations described throughout this thesis constitute my own original work. No part of this thesis has been accepted for any other degree or diploma in any University or College, and to the best of my knowledge contains no copy or paraphrase of material previously published by another person, except where due reference is made in the text.

Signed



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Date: June, 1983

COMMUNICATIONS

Much of the work presented in this thesis and other work not presented herein has been published and presented at scientific meetings as follows.

Publications

1. Woods GM and Lowenthal RM (1981). Red blood cell requirements of human T lymphocyte colonies. J. Clin. Lab. Immunol. 6; 175-180
2. Woods GM and Boyd RL (1981). Contact-dependent expression of actin in chicken lymphocytes in vitro. Int. Archs Allergy Appl. Immunol. 67; 335-339
3. Lowenthal RM, Sawyer PJ, Lickiss JN, Harlow RWH, Kirov SM and Woods GM (1982). Immunological types of lymphoproliferative disorders in a cohort: A 4-year study. Aust. N.Z. J. Med. 12; 258-262
4. Woods GM, Lowenthal RM, Warren HS and Lafferty KJ (1982). Interleukin-2 supports T lymphocyte colony formation. Immunobiology. 163; 398-399 (abstr.)
5. Woods GM and Lowenthal RM. Cellular interactions and IL2 requirements of PHA-induced human T lymphocyte colonies. Submitted manuscript.
6. Woods GM and Lowenthal RM. Low dose irradiation and PHA-induced T lymphocyte colonies: Evidence for a repair enzyme effect. Submitted manuscript.

Abstracts

7. Woods GM and Lowenthal RM (1980). Red blood cell requirements of T-lymphocyte colonies. Abstr. Ann. Meet. Aust. Soc. Immunol., Brisbane, p. 32.
8. Woods GM. and Lowenthal RM (1981). Inhibition of T-lymphocyte colonies by low dose irradiation. Abstr. Ann. Meet. Aust. Soc. Immunol., Canberra, p. 35
9. Woods GM, Lowenthal RM, Warren HS and Lafferty KJ (1982). Interleukin 2 supports T lymphocyte colony formation. Presented at the 15th International Leucocyte Culture Conference, Asilomar, California, December 5-10.
10. Lowenthal RM, Woods GM, Warren HS and Lafferty KJ (1983). Interleukin-2 supports T lymphocyte colony formation. Abstr. Inaugural. Meet. Transplant. Soc. Aust. N.Z., Canberra, p. 8.

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COMMONLY USED ABBREVIATIONS

Ab	antibody
alpha-medium	alpha modified Eagles medium
B-CLL	chronic lymphocytic leukemia of B lymphocytes
D ₀	radiation dose reducing survival to 37%
Con A	concanavalin A
E-rosette	sheep red blood cell rosette
Fc- IgG	receptors for the Fc fragment of an IgG molecule
Fc- IgM	receptors for the Fc fragment of an IgM molecule
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
Gy	Gray (100 rad)
Ig	immunoglobulin
IL1	interleukin 1 (lymphocyte activating factor)
IL2	interleukin 2 (T cell growth factor)
Leu 2a	monoclonal antibody to "suppressor" cells
Leu 3a	monoclonal antibody to "helper" cells
MEM	modified Eagles medium
M-rosette	mouse red blood cell rosette
n	extrapolation number
NK	natural killer cells
OKT3	monoclonal antibody to circulating T lymphocytes
OKT4	monoclonal antibody to "helper" cells
OKT8	monoclonal antibody to "suppressor" cells
PBS	phosphate buffered saline
PHA	phytohaemagglutinin
SRBC	sheep red blood cells
TLCFC	T lymphocyte colony forming cell

REAGENTS AND EQUIPMENT

Reagents:-

acetic acid	Ajax chemicals, Auburn, NSW, Aust.
agar	Bacto-Agar, Detroit USA
alpha-medium	Flow Laboratories, Scotland
cytochalasin B	Sigma, St. Louis, Mo., USA
DMSO	Sigma
Eosin-Y	George T. Gurr, BDH chemicals, Vic., Aust.
FCS	Flow Laboratories
FITC-rabbit- anti-rat-Ig	(MF08) Wellcome, Vic., Aust.
Histopaque	Sigma
Leu 2a & Leu 3a	Becton-Dickinson Monoclonal Center, Mountain View, USA
Liquifluor scintillation fluid	New England Nuclear Boston, Mass., USA
MEM	Flow Laboratories
orcein	George T. Gurr
PBS	Oxoid, Vic., Aust.
PHA	(Reagent grade HA15) Wellcome
polyvalent FITC-Ig	Calbiochem-Behring, NSW, Aust.
SRBC	Commonwealth Serum Labs., Vic., Aust.
trypsin	BDH, Vic., Aust.

Minor Equipment:-

centrifuge tubes	Johns Products, Vic., Aust.
glass coverslips	Vitromed, Basel
glass slides	Corning Glass Works, Corning, NY
lithium heparin Tubes	Johns Products
microtitre trays	Linbro (cat 76-013-05) Flow Laboratories
Millipore membrane apparatus	Millipore Pty. Ltd., North Ryde, Aust
petri dishes (35 x 10 mm)	Lux (cat 5217), Lab-Tek, Naperville, IL, USA
petri dishes (100 x 10 mm)	Disposable Products, SA, Aust.
universal containers	Johns Products

Major equipment:-

Clemco DF-44R Laminar Flow Cabinet
 Forma Scientific Incubator (model 3157)
 Leitz Orthoplan Fluorescence Microscope
 MSE Mistral 4L refrigerated centrifuge
 Olympus dissecting microscope
 Ultrays X-ray machine
 Unicam SP8-100 Ultraviolet spectrophotometer
 Titertek D-001 Cell harvester
 Zeiss light microscope

TO MY WIFE
AND TO MY PARENTS

S E C T I O N O N E

HISTORICAL REVIEW AND OUTLINE OF THESIS

1.1 Introduction

This thesis is essentially concerned with the technique of PHA-induced T lymphocyte colony formation as applied to studies of human T lymphocytes.

1.2 The lymphocyte

On a purely morphological basis the lymphocyte would appear to belong to an unimportant assembly of cells. As a legacy of this simplicity in both size and shape and the apparent insignificance of its functions lymphocyte studies were shelved for at least two centuries. It has only been in the past 20-30 years that the lymphocyte has been considered as central, and therefore vital, to the immune response. The first resolute attempts at studying lymphocytes in vitro were initiated by Ranvier during the middle of the nineteenth century (quoted by Ford, 1980). These initial experiments were designed to determine if lymphocytes demonstrated a motility already shown for phagocytic cells. He concluded that lymphocytes did in fact migrate across a glass slide with an action not unlike that of amoebae and acknowledged the original proposal of Jones (1842). These observations were confirmed by Renaut (1881) but as has become characteristic of many lymphocyte functions these conclusions were contradicted (Ehrlich and Lazarus, 1905; see Maximov, 1932). The controversy over the lymphocytes' motility, or lack of it, dominated research in this area for almost 50 years. The answer was not completely resolved until Lewis and Webster (1921) revealed that defined media and temperature conditions were required to demonstrate motility. In retrospect this controversy seems somewhat prophetic as many lymphocyte functions and characteristics are still controversial

and it would appear that technical considerations need to be accounted for; this is particularly relevant to the culturing of human T lymphocyte colonies. Intense study of the lymphocyte over recent years has revealed it be a cell with a remarkable potential for a wide range of functions. The apparently simple morphological structure belies the heterogeneity of the various lymphocyte subpopulations that have recently been enumerated and their importance in immune reactions cannot be overemphasised. A number of assays have been designed to study lymphocyte functions and relevant to this thesis is the adaptation of the semisolid agar technique of granulocyte/macrophage cultures to the culturing of T-lymphocytes in vitro.

1.3 Soft agar cultures

The need for haematologists to further understand the processes involved in haemopoiesis led to the development of specialised in vitro culture assay systems. Studies with intact animals were extremely difficult due to the highly complicated organization of cells and the immense number of factors that influenced them. Early studies employed a variety of liquid culture systems and although a host of valuable information was derived an understanding of the events of proliferation, regulation and functional activity of individual cells and their progeny could not be obtained. It became clear that alternative techniques were required. The successful cloning of virally transformed fibroblasts in semisolid medium by the tumor virologists (see review by MacPherson, 1970) opened up the exciting prospect of studying clones of individual cells. In this system only virally transformed fibroblasts produced colonies whereas the normal fibroblasts died. This was an appealing

observation and it invited work with leukemic cells with the hope that these cells would grow in contrast to the normal cells and thus providing a discriminating assay for normal and leukemic cells. As reviewed by Metcalf (1977) early attempts at culturing leukemic cells repeatedly failed until feeder layers of cells, preceded by Puck and Marcus (1955) in an entirely different system, was employed. Colony growth was observed but surprisingly it was not the leukemic cells that grew, but the granulocyte/macrophages derived from the feeder cells (Bradley and Metcalf, 1966; Ichikawa, Pluznik and Sachs, 1966). The fact that both granulocytes and macrophages could develop in agar suggested a variety of haemopoietic cells could be cultured in this way. From an analysis of this granulocyte/macrophage system a requirement for diffusible growth factors (initially from the leukemic cells) was inferred. This inspired Axelrad and coworkers to successfully culture virally transformed erythropoietic cells using the purified erythropoietic regulator erythropoietin as such a factor (Stephenson et al., 1971; McLeod, Shreeve and Axelrad, 1974). The first report of human granulocyte/macrophage colonies was published by Pike and Robinson (1970) who used feeder layers to provide the necessary growth factors. Following these initial studies a number of cell types were cultured and the regulatory factors involved were examined. These studies proved crucial as they allowed for the first time the possibility of an extensive dissection of the regulation of haematopoiesis. Although progress in culturing many hemopoietic cells was rapid the successful culturing of T lymphocytes was a stumbling block for many years.

1.4 T lymphocyte colonies

The adaptation of the virally transformed fibroblast cultures to suit granulocyte/macrophage cultures demonstrated that the culture requirements for these two systems were not identical. Consequently it appeared likely that the induction of T lymphocytes to form colonies would require an additional stimulus. The plant lectin phytohaemagglutinin (PHA) was shown by Nowell (1960) to stimulate resting lymphocytes to undergo blast transformation. This fact prompted Rozenszajn and coworkers to attempt colony growth from PHA-treated cells. The success of their initial experiments was first reported in 1973 (Rozenszajn, Kalechman and Shoham, 1973) and 1974 (Rozenszajn, Kalechman and Shoham, 1974) and further substantiated in 1975 (Rozenszajn, Shoham and Kalechman, 1975). Essential for this technique was an initial sensitization or stimulation of the cells with PHA during a liquid culture (the first step). The PHA-sensitized cells were then seeded into 0.32% agar which was layered above a harder (0.5%) agar base containing PHA (the second step). Growth required a continual presence of PHA, was maximal after 6-8 days and a colony was defined as containing 50 or more cells. These results were confirmed by Fibach, Gerassi and Sachs (1976) and by Wilson and Dalton (1976) who also used two step double agar layer systems. At this time two types of colonies were described. Type I colonies grew within the agar matrix and type II colonies grew as flattened colonies near the surface of the agar medium. Type II colonies were more common than type I and were relatively easy to harvest (Fibach et al., 1976).

A one step technique for T colony formation was first reported by Riou et al. (1976). As for the two step procedure PHA was the stimulus and two layers of agar were required to induce colony

formation but the liquid preincubation was omitted, instead the unstimulated mononuclear cells were seeded directly into agar which contained the PHA. It must be noted that some normal subjects failed to produce colonies suggesting the conditions for colony growth might be suboptimal. A further advance in the production of colonies was demonstrated by Claesson et al. (1977a) who found that by incorporating sheep red blood cells (SRBC) and PHA into the agar medium, two layers of agar were not required and the plating efficiency was improved. In addition colonies could be produced without the prior PHA-sensitisation although colony growth was more efficient with the two step system. Most previous reports utilised agar at the concentration of 0.3% whereas Claesson et al. (1977a) found 0.45% was necessary due to the motility of the cells. A further modification was described by Shen et al. (1977) who replaced the agar with methyl cellulose. Already these early reports demonstrated a variety of methods for producing T cell colonies. This trend flourished over the next few years and unfortunately a development of a wide range of culture techniques occurred, which may or may not be comparable insofar as their results are concerned. These variations, summarised in Table 1.1, include stimulation by mitogens other than PHA e.g. concanavalin A (Con A), pokeweed mitogen (PWM) and Protein A or allogeneic stimulation, culturing in microcapillary tubes or microtitre wells, liquid overlayer cultures, the incorporation of numerous sources of conditioned media and the addition of various irradiated or non-irradiated feeder cells. Inherent in all of these techniques was a requirement for stimulation (either mitogen or alloantigen) and the presence of accessory cells or factors. The diversification of these culture techniques is reflected in the

abbreviations used to designate the colony forming cell. These include TLCFC, TCFC, TCPC, TLCFU, CFU-TL and CFU-L (Table 1.1). Furthermore the definitions of a colony demonstrate the independent nature of many reports as 4, 6, 20, 32, 40 and 50 cells have been nominated. Clearly one must have reservations about direct comparisons between the results of these different techniques.

1.5 Nature of the colony forming cell

As initially reported by Rozenszajn et al. (1975) the cells comprising the colonies could be considered as T cells by their ability to form spontaneous rosettes with sheep red blood cells. This observation has had continued support of different workers using a variety of culture systems (Table 1.1). Morphological investigation under light and electron microscopy has revealed colony cells to be mixtures of "lymphoblastoid-like" and "lymphocyte-like" cells (Rozenszajn et al., 1975; Wilson and Dalton, 1976; Goube de Laforest et al., 1979a; Lowenberg and de Zeeuw, 1979; Wilson et al., 1980; Claesson, Sonderstrup-Hansen and Poulsen, 1981). Further support for the T cell lineage was obtained when the presence of human T lymphocyte antigen (HTLA) was demonstrated through fluorescent microscopy (Klein et al., 1981a) and complete abrogation of colony formation when the cells were pretreated with anti-thymocyte serum plus complement (Shen et al., 1977) or an anti-T cell serum plus complement (Mercola and Cline, 1979), anti B-cell serum having no effect (Shen et al., 1977; Mercola and Cline, 1979). At no time, however, has the presence of 100% E-rosettes been reported while reports have shown cells with non-T markers to be amongst the colonies, albeit as a minor proportion (Rozenszajn et al., 1975; Claesson et al., 1977a; Shen et al., 1977; Farcet, Kincade and Moore,

1980a). This suggests that the colonies were either an admixture of T and non-T cells or that, as colony pooling was performed to obtain a sufficient number of cells for analysis, a small proportion of colonies were made up of non-T cells. Although the proportion of these non-T cells was small the fact remains that T cell colonies may not be comprised of only T lymphocytes and the clonality of these colonies needs to be critically considered.

The demonstration of the T cell nature of most colony cells indicates that the colony progenitor cell is either a pre-T cell (analogous to the GM-colony system in which colonies derive from committed stem cells) or a mature T cell since only the latter respond to PHA. Claesson et al. (1977a & 1978), by separating lymphocytes into E-rosette positive and negative fractions found that colony formation was restricted to the positive fraction. In contrast Triebel et al. (1981) presented evidence that blood mononuclear cells depleted of T cells by E-rosetting or lysis with the OKT3 monoclonal antibody (reactive with peripheral blood T lymphocytes) and complement gave rise to colonies that were both ERFC+ and OKT3+ and therefore concluded that the colony progenitor cell was an immature T cell. This finding was not supported by Klein et al. (1981b). Further evidence supporting the mature (ERFC+ or OKT3+) nature has been substantiated by a number of reports (Foa, Lauria and Catovsky, 1980c; Swart and Lowenberg, 1980; Gelfand et al., 1981). It would therefore appear likely that the colony-forming cells are derived from the E-rosette fraction and that these cells give rise colonies of E-rosette positive cells.

With the demonstration that colony progenitor cells belong to the E-rosette-positive fraction of lymphocytes the search for the progenitor cells was directed to the bone marrow, spleen, lymph node, thymus, cord blood and thoracic duct. In addition to peripheral blood, colonies were produced by lymphocytes from all of these sources excluding the thymus and thoracic duct (Rozenszajn et al., 1975; Foa and Catovsky, 1979; Lowenberg and de Zeeuw, 1979; Foa, Catovsky and Lauria, 1980a; Gelfand et al., 1981). Of these only the thymus and thoracic duct populations are comprised of immature lymphocytes and therefore it is highly unlikely that colonies are derived from immature stem cells; a prediction made by a direct analogy to the granulocyte/macrophage system. This was supported by the disclosure that the colony progenitor cells belong to the E-rosette-positive fraction and were resistant to theophylline, a feature of mature lymphocytes (Gelfand et al., 1981). Although bone marrow and cord blood are rich in progenitor cells (Knudtzon, 1974; Gabutti et al., 1975) and relatively depleted of functionally mature cells the formation of colonies was not due to these immature cells but to the residual phenotypically mature T cells. Depletion of E-rosette positive cells removed the colony forming potential (Foa et al., 1980a; Swart and Lowenberg, 1980).

When peripheral blood lymphocytes were fractionated over a discontinuous albumin density gradient and assessed for colony-forming ability and responsiveness to PHA as measured by tritiated thymidine uptake, it was found that the cells forming colonies were a subpopulation of PHA-reactive cells (Swart and Lowenberg, 1980). However, in the bone marrow a proportion of PHA-nonreactive lymphocytes did in fact give rise to colonies (Lowenberg and de Zeeuw,

1979; Swart and Lowenberg, 1980). From this result Lowenberg and de Zeeuw suggested that within the bone marrow the colony progenitor cells may be immature T lymphocytes. This may be an artefact of the thymidine incorporation assay used, because of the immature dividing haemopoietic cells giving an abnormally high value. From E-rosette separation experiments it is apparent that within the peripheral blood and bone marrow compartments the T colony-forming cells comprise a subpopulation of E-rosette positive cells (Swart and Lowenberg, 1980). Studies with human cord blood failed to demonstrate a direct parallel between the number of colonies and the percentage of E-rosette forming cells (Foa et al., 1980a). This poor correlation might be due to a selective subpopulation of T lymphocytes producing colonies. In support of this possibility Claesson et al. (1978) found colony formation was restricted to T lymphocytes that were negative for Fc-receptors of IgG (Fc-IgG). With a different colony assay Foa et al. (1980c) further substantiated this view in showing that colony formation was mainly confined to the Fc-IgM (helper phenotype) population of T lymphocytes, although attention needs to be paid to the possibility of receptor shedding and switching (from Fc-IgG to Fc-IgM; Pichler, Lum and Broder, 1978). Monoclonal antibody analysis was consistent with this observation as most of the colony cells were positive for the markers Leu 3a and OKT4 (which select for the inducer/helper phenotype of T lymphocytes) (Claesson et al., 1981 & 1982 respectively). On the other hand Gelfand et al. (1981) found neither Fc-IgG nor Fc-IgM on the colony cells while Klein et al. (1981a) found that cells with these receptors comprised less than 20% of the colony cells. It again appears that different culture techniques may select for different subpopulations of T lymphocytes.

From all of these reports the evidence points to the majority of T lymphocyte colonies as being of the helper phenotype (ie Fc-IgG negative, OKT4 and Leu 3a positive) although it is unlikely that all colonies derive from an elite subpopulation of T lymphocytes.

1.6 Functional analysis of T lymphocyte colonies

Functional characterization of the T colony cells was initially difficult to perform due to problems in harvesting sufficient cells for quantitative assays. By pooling a number of colonies it was possible to obtain sufficient cells but this approach precluded the assay of cells from a single colony. With the present knowledge and availability of purified interleukin preparations single colonies could be expanded out for such an analysis. The reports to date on pooled colony cells demonstrate that they respond to T cell mitogens and exhibit a restricted cytotoxic capacity (Price, Teh and Miller, 1980; Gelfand et al., 1981; Klein et al., 1981a). Gelfand et al. (1981) demonstrated a response to the T cell mitogens PHA, Con A, PWM, protein A but not to the B cell mitogen STA (formalinized *S. aureus* Cowan I strain). In addition the colony cells responded to, and stimulated allogeneic cells of a mixed lymphocyte culture (MLC) reaction. These functions are characteristic of T cells. Harvesting the colony cells and assaying for helper or suppressor activity was performed by Bockman and Rothschild (1979) who found evidence for both helper and suppressor activities within the pooled colonies. This finding corroborates the possibility that colonies do not consist of a specific or an unrepresentative lymphocyte subpopulation. When the colonies were pooled and reseeded as single cell suspensions into agar medium they formed new colonies provided a source of accessory cells was present (Goube de Laforest et al., 1979a; Gelfand et al., 1981)

There was, however, a low plating efficiency suggesting that colony regeneration is a property of a small proportion of colony cells. In a similar subculture study it was found that only 1 cell in any given colony (at most) was able to regenerate a new colony (Smith and Sachs, 1979). These workers proposed that this single cell was the only cell within the colony that retained the colony forming potential. In light of recent results concerning the requirement for cellular interactions and factors this observation could be explained by the need for accessory factors.

Assays of the colony cells for cytotoxicity have produced conflicting results. Gelfand et al. (1981) and Price et al. (1980) suggested that the cells undergo a specificity-restricted spontaneous, but not an antibody dependent, cytotoxicity. This is partly contradicted by Klein et al. (1981a) who found that in addition to restricted spontaneous cytotoxicity the colony cells could also lyse antibody coated target cells. The demonstration that pooled colony cells exhibit a limited form of spontaneous cytotoxicity raises the possibility that natural killer (NK) cells produce their own colonies. As NK cells are weakly positive for sheep red blood cell receptors it is difficult to totally exclude their presence. Price et al. (1980) claimed that it is unlikely that colony cells are NK cells because they did not contain Fc-IgG receptors nor did they lyse K562 cell lines. Furthermore Claesson et al. (1981 & 1982) found that the colony cells usually marked with either Leu 3a or OKT4. If NK cells are present it is unlikely that they occur in large numbers.

1.7 Single or multicellular origin of T lymphocyte colonies

A direct comparison with the granulocyte/macrophage or erythrocyte colony systems which, given certain restrictions, have been shown to be clonal (Strome, Mcleod and Shreeve, 1978; Singer et al., 1979), would lead one to suspect that T lymphocyte colonies may derive from single cells and could therefore be clones. There are, however, major differences between the systems with the most salient feature being the maturity of the cell from which the colonies develop. T cell colonies require mature cells, whereas the other hemopoietic colonies develop from immature stem cells. To investigate the question of clonality basically two techniques have been employed. As shown in Table 1.1 the clonal nature is either supported or refuted according to the technique used. By sequential microscopic observation the multiplication of a single cell can be followed. Through this method it was claimed that colonies arose from a single cell (Rozenszajn et al., 1975; Spitzer et al., 1980; Wilson et al., 1980). Although regular observations may allow a satisfactory study of cellular proliferation it does not entirely overcome the criticism that some aggregation may occur as lymphocytes have been shown to demonstrate significant migration even in very high (0.8%) agar concentrations (Lindahl-Kiessling, Clarke and Riddle, 1982; Lindahl-Kiessling and Karlberg, 1982).

The alternative method to test the multi- or unicellular models was to analyse glucose-6-phosphate dehydrogenase (G6PD) from females heterozygous for the A and B iso-enzymes. The principle behind this test is that some women have two distinguishable isoenzymes (A and B) which are coded on the X chromosome and therefore random

inactivation of one of these isomers will occur. Consequently a single cell will express only one of these isoenzymes (A or B); this can be detected electrophoretically. Analysis of single colonies will determine the presence of either or both of the isomers thereby demonstrating uni- or multicellular origins respectively. The initial report by Gerassi and Sachs (1976) supported a clonal nature for the colonies. However in this report cells were pooled from a number of females who differed in this enzyme rather than cells from a heterozygous female. These cells were cocultured and the colonies were analysed for G6PD. The disadvantage with this method was that cells from one individual could aggregate prior to cell division and therefore the origin could not be determined. Furthermore their data indicated that some (7/28) colonies originated from more than one cell. Later reports with single donors revealed heterogeneity of the enzyme isomers within single colonies thereby supporting a multicellular origin (Singer et al., 1981; Farcet and Testa, 1982). This was demonstrated with single and double-step procedures and with low (0.3%) and high (0.45%) agar concentrations (Singer et al., 1981). By analysing the number of colonies with both isoenzymes Singer et al. (1981) concluded that, for the two step procedure, a colony had to be derived from at least 10 cells. This prediction was not borne out when the cells were observed microscopically as groups of no more than 4 cells could be seen 3 days after the culture had commenced. A similar enzyme analysis was performed by Farcet and Testa (1982) who concluded through statistical analysis that colonies resulted from the proliferation of two cell populations. This is consistent with the concept that cellular cooperation enabled colonies to grow; however proliferation of accessory cells may not be essential (Zeevi, Goldman

and Rozenszajn, 1977; Goube de Laforest et al., 1979a; Gelfand et al., 1981; Klein et al., 1982). Aside from enzyme analysis evidence for the polyclonal origin of the colonies was obtained when single colony cells were analysed for surface membrane markers (Farcet et al., 1980b). At different stages of colony formation the presence of macrophages, T cells, B cells and plasma cells was demonstrated. Furthermore, from the ability of T cells to migrate in agar medium Lindahl-Kiessling and Karlberg (1982) found that colonies developed through recruitment of mobile cells as well as division within the colonies.

It is clear that the demonstration of either the multi- or uni-cellular origins will depend on both the culture techniques and the methods used for analysis. It must be realised that accepting one theory for one culture system neither refutes nor supports another theory for another culture system. As previously discussed a number of different techniques have evolved for the production of T colonies. Before the question of the clonal origin of T lymphocyte colonies can be addressed a number of points should be considered. Firstly an interpretation of the kinetics of cell dose response curves will reveal whether a clonal nature is possible. If the curve is linear and passing through the origin then there is no evidence for cellular aggregation and a clonal nature is possible. If the curve is exponential or linear but not passing through the origin, then this may be taken as evidence that a minimum number of cells are required to bring about colony development. Agar concentration is also important. As previously discussed agar concentrations vary from 0.3% to 0.45% and colonies have also been cultured in liquid overlays. Clearly the lower the agar concentration the more likely it is that

cellular aggregation will occur. Therefore the question of agar concentration is important with respect to the types of colonies assessed. As previously discussed type I and type II colonies have been described and it has mainly been the type-II colonies that have been analysed. As these colonies are found in liquid cultures or on the surface of low concentration (0.3%) agar it is likely that these cells are mobile and therefore aggregate because of the agglutinating properties of PHA. Type I colonies have not specifically been studied due to the difficulty of harvesting the cells. These colonies would not have this agglutinating property and may in fact be clonally derived. This is in agreement with time sequence observation studies that did not demonstrate aggregation at early stages of colony growth. However Singer et al. (1981) studied both surface (type II) and internal (type I) colonies and claimed that both types of colonies were heterogeneous. The final point to consider is the exact culture technique used. There is strong evidence suggesting that a colony of T lymphocytes requires the cooperation of a number of accessory cells; these cells may be incorporated into the colony although they may not undergo cell division themselves. With these points in mind it is highly improbable that the colonies previously assessed for monoclonality can arise from a single cell. Because of these findings the possibility that a single T cell can divide and form a true clone is not ruled out provided a number of conditions are met. These will include a suitable agar concentration to prevent excessive cell migration without affecting viability, a replacement of helper cells with the relevant helper factors (e.g. interleukin 1 and interleukin 2) to alleviate the need for cellular interactions and the plating of sufficient cells to prevent cell crowding. The fact that the reports

already described favour a polyclonal origin for T lymphocyte colonies does not invalidate the technique but merely adds the qualification that experiments based on cellular cooperation would be more suited to the T colony system as the production of a colony measures the effective interactions of a number of cell types.

1.8 Cellular interactions and colony formation

It was becoming increasingly evident that cooperation between different cell populations was essential for T colony development (Zeevi et al., 1977; Goube de Laforest et al., 1979a; Gelfand et al., 1981; Klein et al., 1982). Evidence was mounting that cooperating cells exerted their influence through the elaboration of diffusable factors from particular cell populations (Claesson et al., 1977b; Zeevi et al., 1977 & 1978; Gerassi and Sachs, 1978; Foa, Zafar and Catovsky, 1980d; Rozenszajn et al., 1981). The exact nature of these cells still requires clarification as a number of cell types has shown some form of colony promoting ability. Included are T cells (Zeevi et al., 1977), B cells (Klein et al., 1982), monocytes (Claesson et al. 1977b) and even malignant B cells (Douer and Sachs, 1979a). Humoral factors have been shown to inhibit colony growth (Spitzer et al., 1980; Claesson et al., 1981; Klein et al., 1981b; Rozenszajn et al., 1981) and it would therefore appear likely that colony formation is controlled by both positive and negative influences. It would be reasonable to anticipate that monocytes and suppressor T cells negatively influence colony growth. There have been various reports supporting this view for the monocytes (Zeevi et al., 1977; Spitzer et al., 1980; Rozenszajn et al., 1981; Klein et al., 1982), however the role of suppressor T cells is unclear. It has been shown through mixing experiments that T cells with Fc-IgG receptors

(suppressor cells) inhibit colony formation (Canonica et al., 1980; Claesson et al., 1981; Nemoto, et al., 1982). In a later report Claesson et al. (1982) found that OKT8+ cells inhibited the colony formation of OKT4+ cells. On the other hand Foa et al. (1980c) did not find any colony suppression when suppressor cells and normal lymphocytes were cultured.

1.9 Applications of T lymphocyte colony assays

The potential of the T colony culture system as an index of immunological activity was first recognised by Wilson and Dalton (1976). A depression of T lymphocyte colony formation was seen in all 17 cancer patients tested compared to the controls. This was further substantiated when 55/62 patients with a wide variety of malignant disorders were also found to have a significantly reduced colony forming ability (Sutherland, Dalton and Wilson, 1976). The depression of T lymphocyte colony formation was more consistent and more significant than assays of skin tests to 5 recall antigens or thymidine incorporation by PHA stimulated cultures from the same peripheral blood lymphocytes. This tends to suggest that the T colony assay is a sensitive technique for investigations into immune disorders.

Patients with lymphoproliferative disorders commonly have an impaired cell mediated immunity. When lymphocytes from such patients were analysed for colony formation a severe to complete inhibition was observed (Dao et al., 1978; Foa and Catovsky, 1979; Foa et al., 1980b; Pistoia et al., 1982). An important observation was that normal colony growth was obtained with lymphocytes from acute leukemia patients in complete remission (Foa and Catovsky, 1979). In B

cell chronic lymphocytic leukemia (B-CLL) normal T cells are overwhelmed by the high proportion of malignant B cells. When unseparated mononuclear cells from these patients were assayed for colonies a significant reduction was observed (Dao et al., 1978). This is not an unexpected finding in view of the low numbers of T cells plated due to the dilution effect caused by the malignant B cells. When these malignant B cells were removed Douer and Sachs (1979a) found that the residual T cells produced normal colony numbers. Furthermore the leukemic B cells were beneficial as they produced some form of colony enhancing activity (Douer and Sachs, 1979b). This "normal" ability of the T cells to produce colonies was not supported by Foa et al. (1980b & 1982) who found some degree of impaired colony formation. As a compromise Pistoia et al. (1982) proposed that certain B-CLL patients were unresponsive in the colony forming assay while other patients were apparently normal. This conflict may merely reflect the diversity of the disease.

Patients with Hodgkin's disease have impaired cell mediated immunity (Corder et al., 1972; Eltringham and Kaplan, 1973; Levy and Kaplan, 1974; Winkelstein et al., 1974; Hillinger and Herzig, 1978). As anticipated T colony formation from these patients was reduced (Bockman, 1980; Douer and Sachs, 1979a; Dao et al., 1978) and there was a progressive reduction in colony formation with advancing stages of the disease (Bockman, 1980). Patients in apparent remission did not return to a normal colony forming ability (Douer and Sachs, 1979a) suggesting some form of immunodeficiency persisted during this stage.

In cases of non-malignant disorders associated with immune suppression, T colony formation is also depressed. Patients with the auto-immune disease systemic lupus erythematosus (SLE) have a number

of impaired T cell functions (Toh et al., 1973; Horwitz and Cousar, 1975). Defects in colony formation have been reported in such patients (Whittingham et al., 1978; Bernstein et al., 1980) which corresponded to the severity of the disease (Eckels and Gershwin, 1981). The reduction was more marked than in the other tests for cell mediated immunity including the responses of blood lymphocytes to PHA in liquid culture, the number of circulating T cells and serological abnormalities (Bernstein, Winkelstein and Dobson, 1980). The suggestion that T colony formation was a sensitive marker of cell mediated immunity is further qualified by the colony experiments on lymphocytes obtained from infectious mononucleosis patients. The depression of colony formation was most severe at the early stages of the disease corresponding to the active period but returned to normal levels during the convalescing periods (Claesson and Anderson, 1979).

It has been established that aged persons have a slight but detectable reduction in cell mediated immunity (Pisciotta et al., 1967; Fernandez, MacSween and Langley, 1976; Fetisova, 1978; Hallgren et al., 1978). T colony formation was reduced in elderly people (Whittingham et al., 1978; Rozenszajn et al., 1981; Alder, Morley and Seshadri, 1982), again suggesting a role for T colony growth as a sensitive measure of cell mediated immunity. This proposal was substantiated by Herrod and Valenski (1981) who obtained peripheral blood lymphocytes from 12 immunodeficient patients and found that the reduction in colony formation was more sensitive than either E-rosette formation or blast transformation. It would therefore appear likely that the T colony assay system is ideally suited for studying cell mediated immunity and the factors (both enhancing and inhibitory) that influence such an immunity.

The development of an in vitro system for the growth of human T lymphocytes was an extension of the well established haemopoietic colony techniques. The early difficulties in culturing human T lymphocytes in semisolid agar were overcome when it was realised that PHA-stimulated lymphocytes possessed the ability to form colonies. Since the initial reports describing T colony growth were made many technical variations have evolved; prerequisites for all being a stimulatory signal (i.e. in the form of mitogen or alloantigen) and accessory cells or factors (e.g. irradiated leucocytes or conditioned medium). The T cell nature of the colonies was verified in many independent reports although none showed that all colonies comprise 100% T lymphocytes. In fact, as it is likely that colony formation requires cooperation with accessory cells it is also likely that colonies are not clonally derived. Such a conclusion was asserted by those investigators who specifically addressed this question. Studies on the colony cells have shown that they mainly express T cell characteristics i.e. they respond to T cell mitogens, react in a mixed lymphocyte culture and exhibit a restricted cytotoxic capacity.

Analysis of colony growth from immunodeficient patients, both malignant and non-malignant, revealed a dramatic reduction in the number of colonies produced. The results from these studies pointed to the T colony assay as a sensitive marker of cellular immunity and with the evidence that cellular cooperation was vital it seemed that the production of a colony was the "end-product" of a number of events. Consequently it appeared likely that the T colony assay could be used for in vitro assessment of the events of cell mediated immunity.

The initial aim of this thesis was to devise an improved method for the culturing of human T lymphocytes in semisolid agar. As discussed above a number of techniques were becoming available. At the initiation of this study the method described by Claesson et al. (1977a) seemed the most suitable. Section 2 of this thesis therefore describes the initial experiments performed to obtain the most beneficial method for this laboratory. In section 3 the colonies were analysed in further detail. An interesting but essential requirement for colony growth was a source of sheep red blood cells. The reasons as to why these cells were needed were examined in section 4. Indirect evidence was accumulating (from this study and those of other workers) that colony growth required the collaboration of accessory population(s) of "helper" cells. It appeared likely that adherent cells (monocytes) would be a prime candidate to fill the role of accessory cell. Section 5 considers the dependence of colony formation on a source of adherent cells. Admittedly the field of monocyte/lymphocyte interactions is extremely diverse and no attempt was made to encompass the entire field. It should therefore be noted that the single aim of this particular section was to determine if adherent cells were involved in the production of colonies and if so at what stage were they essential. In section 6 the role of IL2 (more specifically a lymphokine with IL2 properties) in colony formation was examined. The final results section of this thesis (section 7) utilised the T colony assay to study the effects of irradiation on T lymphocyte cell division with particular emphasis on the radio-protection afforded through PHA-stimulation. Finally section 8 discusses these results in relation to those of other investigators.

Table 1.1

Summary of the more important features described for T lymphocyte colony formation reported in the literature

CULTURE TECHNIQUE				COLONIES				REFERENCE
indirect or direct	double or single	conc. agar %	min. size	range	cells seeded	colony abbrev.	other	
indirect	double	0.32	50	n.s.	n.s.	n.s.	1 2 10 13	Rozenszajn et al., 1975
indirect	double	0.33	50	345- 2383	500,000	n.s.	4 10 13	Gerassi and Sachs, 1976
indirect	double	0.30	50	1050- 4000	1,000,000	n.s.		Sutherland et al., 1976
indirect	double	0.33	50	391- 591	500,000	n.s.	1 4 13	Fibach et al., 1976
indirect	double	0.30	50	1150- 2500	1,000,000	n.s.	1 10	Wilson and Dalton, 1976
direct	double	0.30	50	0- 1300	200,000	n.s.	1 13	Riou et al., 1976
indirect	single	0.45	50	n.s.	n.s.	n.s.	1 2 12	Claesson et al., 1977a
direct	single	0.45	4	n.s.	n.s.	n.s.	5 12	Claesson et al., 1977b
direct	single	methy cellulose	40	700- 1000	1,000,000	n.s.	1 3 13	Shen et al., 1977
indirect	double	0.32	50	90- 120	100,000	n.s.	2 5	Zeevi et al., 1977
indirect	n.a.	0.30	50	n.s.	n.s.	n.s.	7	Maurer et al., 1977

Table 1.1 (continued)

CULTURE TECHNIQUE				COLONIES				REFERENCE
indirect or direct	double or single	conc. agar %	min. size	range	cells seeded	colony abbrev.	other	
direct	double	0.33	50	2973- 6850	1,000,000	n.s.	4 5 13	Sachs, 1978
indirect	single	0.45	50	21- 127	1,000E-RFC	n.s.	12	Whittingham et al., 1978
indirect	single	methy cellulose	50	42- 206	100,000	n.s.	12	Dao et al., 1978
direct	double	0.33	50	n.a.	n.a.	n.s.	5 6 13	Gerassi and Sachs, 1978
indirect	double	0.30	50	n.a.	n.a.	n.s.	1 4	Shibasaka et al., 1978
direct	n.a.	0.20	50	20- 50	10,000	TLCFU	1 7	Ulmer and Flad, 1979
direct	double	0.30	6	0.8- 42	100*	n.s.	1 2 10	Mercola and Cline, 1979
direct	single	0.45	32	0- 480	2,000	n.s.	5 13	Claesson and Anderson, 1979
direct	double	0.30	40	367- 2222	250,000	TCFC	1 5	Goube de Laforest et al., 1979a & b
direct	double	0.30	50	684- 5493	1,000,000	n.s.	1 5	Bockman and Rothschild, 1979

Table 1.1 (continued)

CULTURE TECHNIQUE				COLONIES				REFERENCE
indirect or direct	double or single	conc. agar %	min. size	range	cells seeded	colony abbrev.	other	
direct	double	0.33	50	620- 3710	500,000	n.s.	1 5 13	Douer and Sachs, 1979
indirect	single	0.36	10	800- 2400	1,000,000	n.s.	1 2 12	Kornbluth et al., 1979
direct	double	liquid	50	102- 270	100,000	TLCFC	1 6 13	Foa and Catovsky, 1979
direct	double	liquid	50	n.s.	n.s.	n.s.	1 6 13	Lowenberg and de Zeeuw, 1979
direct	double	0.33	50	n.s.	n.s.	n.s.	2 13	Smith and Sachs, 1979
indirect	single	0.50	40	80- 3361	1,000,000	n.s.	1 11 13	Wilson et al., 1980
direct	double	liquid	50	100- 190	100,000	n.s.	6 13	Foa et al., 1980a
direct	double	liquid	50	113- 262	100,000	n.s.	1 5 6 13	Foa et al., 1980c
direct	double	liquid	50	61- 150	100,000	TLCFC	5	Gordon et al., 1980
indirect	double	0.30	50	1914- 2634	1,000,000	n.s.	5	Bockman, 1980
direct	double	0.30	40	0- 3666	500,000	n.s.	3 5 10	Spitzer et al., 1980
direct	double	0.33	10	5669- 6391	750,000	n.s.		Bernstein et al., 1980

Table 1.1 (continued)

CULTURE TECHNIQUE					COLONIES			REFERENCE
indirect or direct	double or single	conc. agar %	min. size	range	cells seeded	colony abbrev.	other	
direct	double	liquid	50	n.s.	n.s.	TLCFC	1 6	Swart and Lowenberg, 1980
direct	single	0.40	n.s.	1370- 4223	1,000,000	n.s.	3 4 5 9	Farcet et al., 1980
direct	double	liquid	50	142- 270	100,000	n.s.	6	Neri et al., 1980
indirect and direct	double	0.30	40	n.a.	n.a.	n.s.	1 9	Singer et al., 1981
	single	methyl cellulose	40	748- 1560	100,000	n.s.	1	Herrod and Valenski, 1981
direct	single	0.30	40	716- 978	1,000,000	CFU-TL	12	Eckels and Gershwin, 1981
direct	single	methyl cellulose	20	223- 459	50,000	TCP	1 5 6 8	Gelfand et al., 1981
direct	double	liquid	50	29- 110	100,000	TLCFC	5	Gordon et al., 1981
direct	single	0.45	32	68- 158	5,000	TLCFC	5 12	Claesson et al., 1981
direct	double	0.30	50	n.a.	n.a.	CFU-TL	4 5 6 13	Rozenszajn et al., 1981
direct	single	0.40	50	n.s.	n.s.	CFU-L	5	Ferrini et al., 1981

Table 1.1 (continued)

CULTURE TECHNIQUE					COLONIES			REFERENCE
indirect or direct	double or single	conc. agar %	min. size	range	cells seeded	colony abbrev.	other	
direct	single	0.30	20	2- 269	15,000	TCFC	5 8	Klein et al., 1981a & b
direct	double	liquid	50	n.s.	n.s.	TLCFC	5	Gordon, 1981
direct	single	0.30	40	n.a.	n.a.	n.s.	1 4 5	Knox et al., 1981
direct	double	varied	50	n.s.	n.s.	n.s.	1 2 8	Konwalinka et al., 1981
indirect	single	0.50	50	123- 511	50,000	n.s.	1 2 12	Woods and Lowenthal, 1981
direct	double	0.30	20	n.a.	n.s.	TCFC	5 8	Klein et al., 1982
direct	double	varied	5	n.a.	n.a.	n.s.	5 9 13	Lindahl-Kiessling and Karlberg, 1982
direct	single	0.20	50	n.a.	n.a.	TLCFU	4 5 7	Ulmer et al., 1982
direct	single	0.36	20	1430- 4070	100,000	n.s.	2 6	Alder et al., 1982

LEGEND

- 1 T cell nature of the colonies demonstrated
- 2 Linear cell dose response curves
- 3 Non-linear cell dose response curves
- 4 Mitogens other than PHA used to induce colony formation
- 5 Accessory cells involved (positive or negative) in colony formation
- 6 Accessory cells or conditioned medium included in culture assay
- 7 Cultures performed in microcapillary tubes instead of petri dishes
- 8 Cultures performed in wells of microculture plates instead of petri dishes
- 9 Polyclonal nature of colonies claimed
- 10 Clonal nature of colonies claimed
- 11 Mixed lymphocyte stimulation used instead of PHA
- 12 Colonies were mainly of the type I category (growing within the agar)
- 13 Colonies were mainly of the type II category (growing on the surface of the agar)

n.s. not stated

n.d. not done

n.a. not applicable

* data not displayed, value described is a standardised frequency value

It should be noted that on a number of occasions the total number of colonies were interpreted from the data included with the published reports and were often obtained directly from the cell dose response curves.

S E C T I O N T W O

PRODUCTION OF HUMAN T LYMPHOCYTE COLONIES

As discussed in section 1 the original methods for culturing human T lymphocyte colonies were two step procedures; a liquid preincubation followed by cultures of either single or double agar layers. The initial report of Claesson et al. (1977a) utilised a two step single agar layer technique. The plating efficiency of this procedure appeared to be superior than most other methods. In addition the colonies were more likely to be derived from single cells because the extremely large colonies produced with other techniques probably resulted from the aggregation of smaller colonies. For these reasons the method of Claesson et al. (1977a) was used as a starting point for studying the growth of human T lymphocyte colonies. Although this method had potential for use as a single step procedure (with smaller colonies and a lower plating efficiency) the two step procedure was pursued because the individual stages of T colony production could be more adequately studied.

This section describes the more salient features arising from the initial culturing experiments designed to ascertain the most favourable conditions for culturing T lymphocytes (in this laboratory). Initially a standard technique was employed (Claesson et al., 1977a) and once established a number of the conditions were sequentially altered. The results described in this section are representative of a number of experiments. In addition to measurable characteristics, certain subjective observations were made (e.g. size of colonies, "health" of colonies etc) which could not be conveyed in figure or table form and therefore the results described herein should be used as a guide to the overall trend rather than exact quantitation.

Fig 2.A

Diagrammatic summary of the T lymphocyte
colony assay as detailed in section 2.2.

30 min
RT
400 g

↓

2 x 10⁶ mononuclear cells
40ug PHA
2 ml alpha-MEM
5% FCS

10% CO₂/air

37°

20 h

50 000 PHA-treated cells
0.5% agar
1% SRBC
20 ug PHA
1 ml alpha-MEM
5% FCS

10% CO₂ / air 37°
7 d

↓

2.2 METHODS

2.2.1 Blood

Peripheral blood was obtained from normal volunteers (laboratory staff or blood bank donors) and was collected into 10 ml heparinized plastic tubes containing 125 i.u. lithium heparin. The heparinized blood was diluted with sterile phosphate buffered saline (PBS) immediately prior to mononuclear cell separation. All blood samples were held at room temperature prior to use.

2.2.2 Lymphocyte isolation

Heparinized peripheral blood was diluted 1:2 with sterile phosphate buffered saline (PBS) and gently layered onto either 3 ml or 10 ml Histopaque 1077 (Isopaque Ficoll) in 10 ml centrifuge tubes or 25 ml universal containers respectively. The preparations were centrifuged at 400 x g for 30 min at room temperature (Boyum, 1968) after which time the centrifuge was gently stopped to prevent gradient disruption. The mononuclear band was harvested and the cells were washed 3 times (once with PBS and twice with MEM) by centrifuging at 200 x g for 10 min at room temperature. Cell counts were performed according to Hudson and Hay (1976). Viability was determined by Eosin dye exclusion.

2.2.3 Culture technique

Using the method described by Claesson et al. (1977a) as a starting point the following procedure was devised and is summarised in Fig 2.A.

2.2.3.1 Preincubation culture

Two million viable mononuclear cells were suspended in 10 ml polystyrene culture tubes in 2 ml alpha-MEM supplemented with 10% FCS and 40 ug PHA. This suspension was incubated for 20 h at 37°C in a humidified atmosphere of 10% CO₂ in air. The cells were washed three times with alpha-MEM and vigorous vortexing was used between each wash to disrupt PHA-induced cell aggregates. In developing this part of the method the following conditions were varied:-

FCS (0 to 20%), cell concentration (1×10^6 /ml to 10×10^6 /ml), PHA concentration (5 - 250 ug/ml) and length of culture (1 - 48 h).

2.2.3.2 Agar culture

Two ml double-concentrated alpha-medium (i.e. medium made up in half the required volume of distilled water) supplemented with 20% (or 10% depending on the batch) FCS was added to sterile glass tubes. To these tubes 40 ug PHA was added. Then, in the following order, 2 ml 1.08% molten agar (held at 40 - 42°C), 0.2 ml PHA-treated cells at required concentration (usually 1.1×10^6 /ml to obtain a final concentration of 50×10^3 /ml) and 0.1 ml 50% (v/v) thrice washed SRBC were added. This liquid agar medium was thoroughly mixed and 1 ml was added to 35 mm polystyrene culture dishes. The agar was allowed to solidify at room temperature prior to incubating the cultures for 7 days at 37°C in a fully humidified atmosphere of 10% CO₂ in air. This allowed for final concentrations of 0.5% agar, 20 ug PHA/ml, 1% SRBC (v/v of packed cells) and 50 000 PHA-treated cells. In developing this part of the method the following conditions were varied:-

FCS (0 to 20%), agar concentration (0.3 to 0.75%), PHA concentration (5 ug/ml to 250 ug/ml), SRBC concentration (0 to 5%) and length of culture (2 - 14 days).

2.2.4 Scoring of colonies

The SRBC's were lysed with either 0.5 ml 2% acetic acid or, if the cells were to be harvested 1.0 ml warm tris-NH₄Cl. Red cell lysis was necessary because the blanket of red cells obscured the colonies. Colonies were scored with a dissection microscope at 20 to 40 X magnification using transmitted light with a dark background. Discrete groups of 50 or more cells were classified as colonies while 10 - 49 cells were classified as clusters. Where possible the entire plate was counted, however, when high colony numbers made this impossible at least 1/4 of the plate was scored. Care was taken to score a representative portion as the edges of the plates contained larger volumes of agar and therefore a higher proportion of the colonies.

2.3 RESULTS

Sections 2.3.1 - 2.3.7 inclusive outline the results of preliminary experiments that were necessary to adopt the culture technique described in section 2.2.3 and summarised in Fig 2.A.

2.3.1 PHA concentration

2.3.1.1 Variation during preincubation culture

The concentration of PHA during the preincubation culture was not critical because a wide range of concentrations was equally effective in permitting colony development (Fig 2.1). On rare occasions colony growth was observed when PHA was omitted from the preincubation culture, however, the colonies were small and not very numerous. The range over which PHA was effective in allowing maximum colony production was 10 to 100 ug/ml and the concentration of

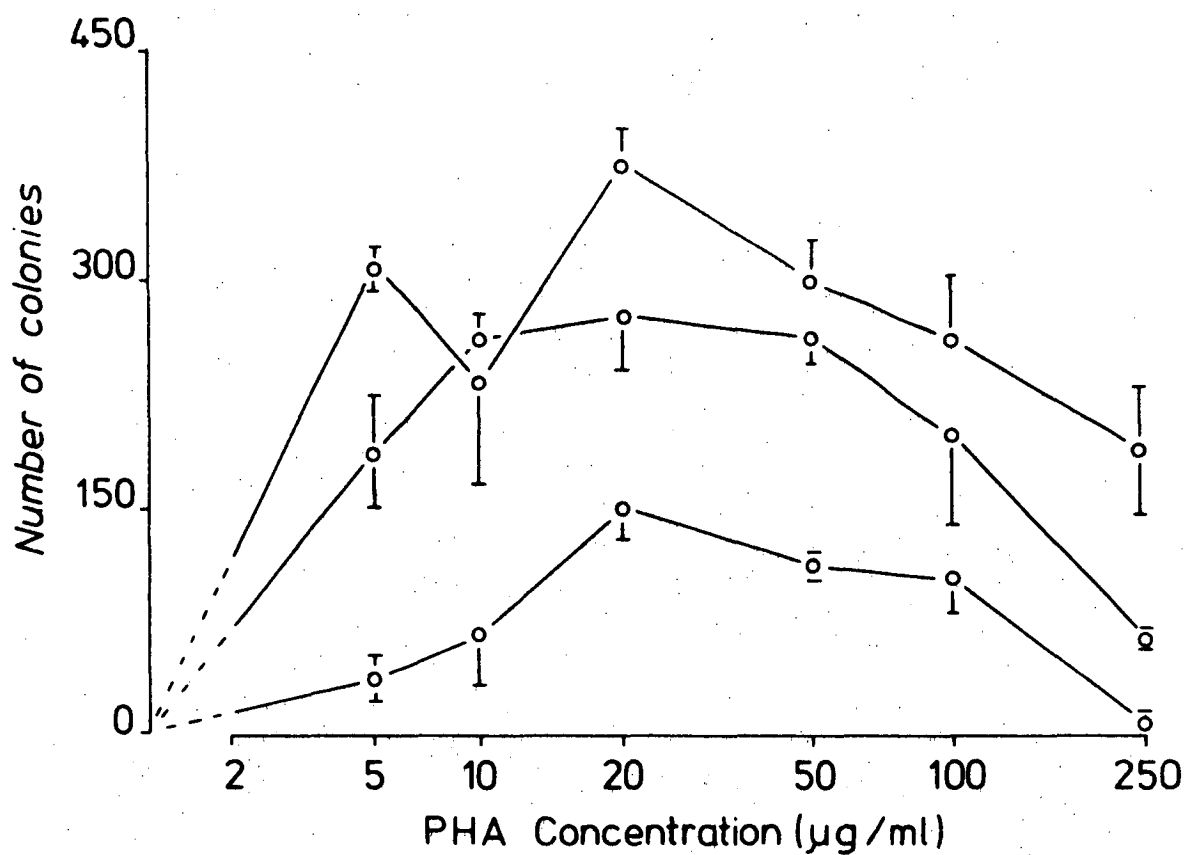


Fig 2.1

Effect of varying PHA concentration during the preincubation culture. One million mononuclear cells were incubated with PHA for 18 h prior to seeding into agar medium containing 20 µg PHA. Each point represents the mean number of colonies from triplicate cultures \pm the standard deviation about the mean when 50 000 cells were seeded into agar. Results from three independent experiments are shown.

20 ug/ml was used in subsequent experiments.

2.3.1.2 Variation during the agar culture

Unlike the previous section the concentration of PHA was critical with optimum activity being restricted to a narrow range (20 to 50 ug/ml) and showing slight variations between different individuals (Fig 2.2). On most occasions there was very little difference between 20 and 50 ug/ml but, as shown in Fig 2.2, 50 ug/ml occasionally produced low numbers of colonies and for this reason 20 ug/ml was chosen as the final concentration for subsequent experiments.

2.3.2 Length of preincubation culture

The optimum preincubation (sensitisation) culture was found to be at least 15 h; longer incubations did not significantly improve colony growth (Fig 2.3). For convenience, preincubation cultures of approximately 20 h (18 - 22 h) were routinely used.

2.3.3 Cell concentration during preincubation culture

At both low and high cell concentrations during the preincubation culture colony growth was poor when both number (Fig 2.4) and size of colonies were considered. As the optimum concentration range appeared to be $1-2 \times 10^6$ /ml all future preincubation cultures were performed with concentrations of approximately 1×10^6 /ml in a maximum volume of 2 ml. When a large quantity of PHA-treated cells was required for seeding into agar a number of preincubation cultures was performed and the PHA-treated cells were pooled.

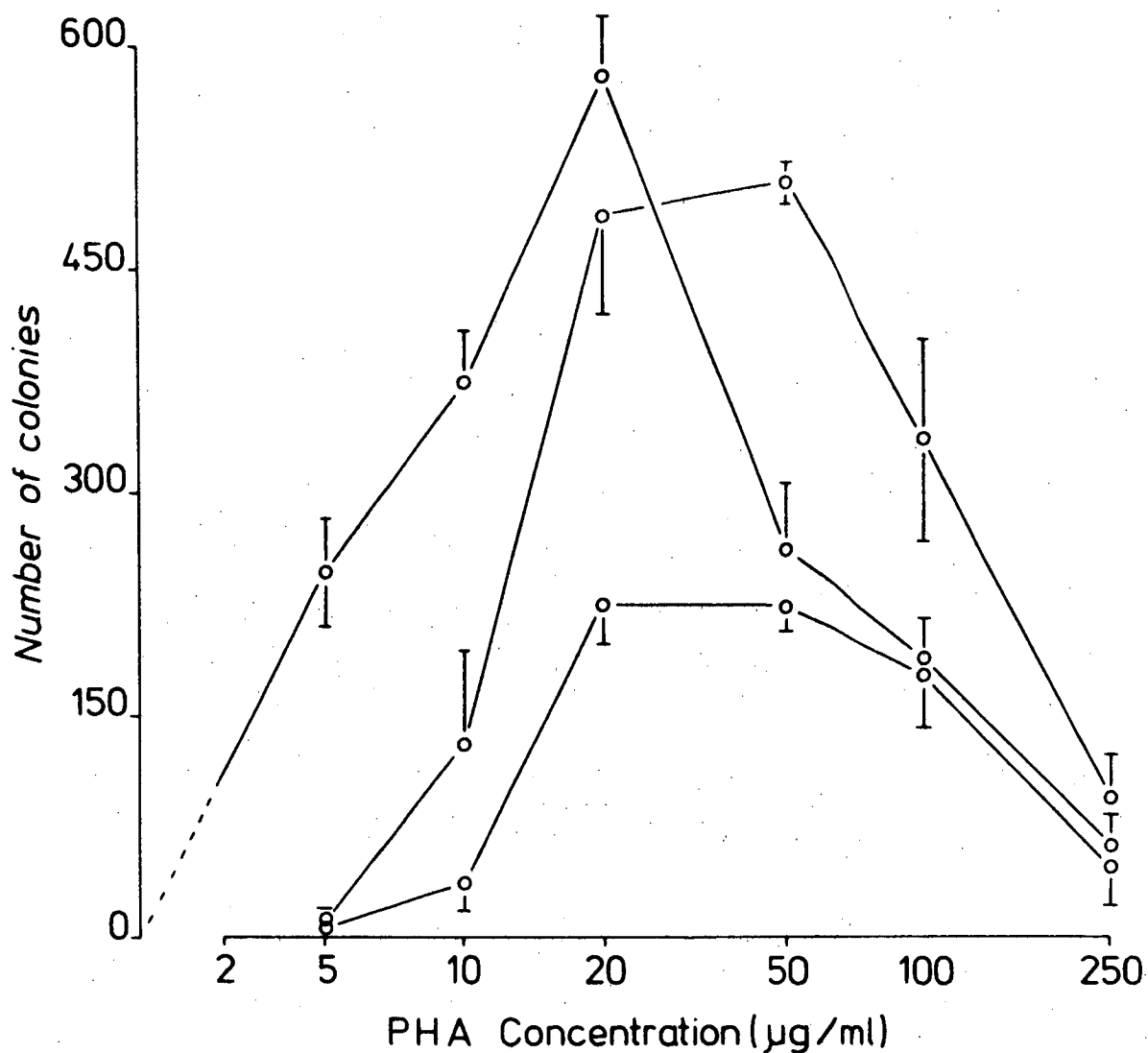


Fig 2.2

Effect of varying PHA concentration during the agar culture. One million mononuclear cells were incubated with 20 ug PHA for 18 h prior to seeding into PHA-containing agar. Each point represents the mean number of colonies from triplicate cultures \pm the standard deviation about the mean when 50 000 cells were seeded into agar. Results from three independent experiments are shown.

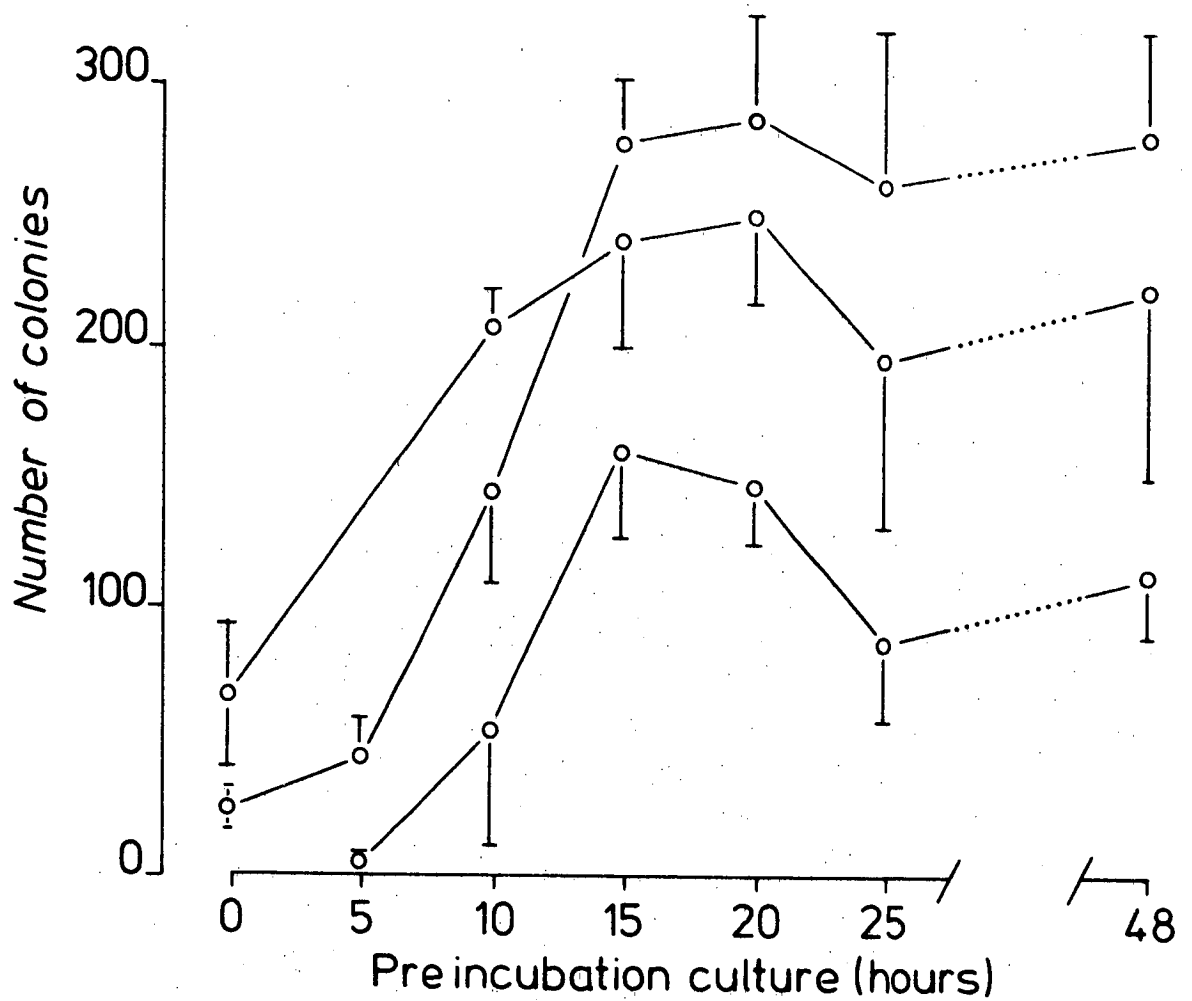


Fig 2.3

Effect of varying the length of the preincubation culture. Mononuclear cells were incubated for various time intervals prior to seeding into PHA-containing agar. Each point represents the mean number of colonies from triplicate cultures \pm the standard deviation about the mean when 50 000 cells were seeded into agar. Results from three independent experiments are shown.

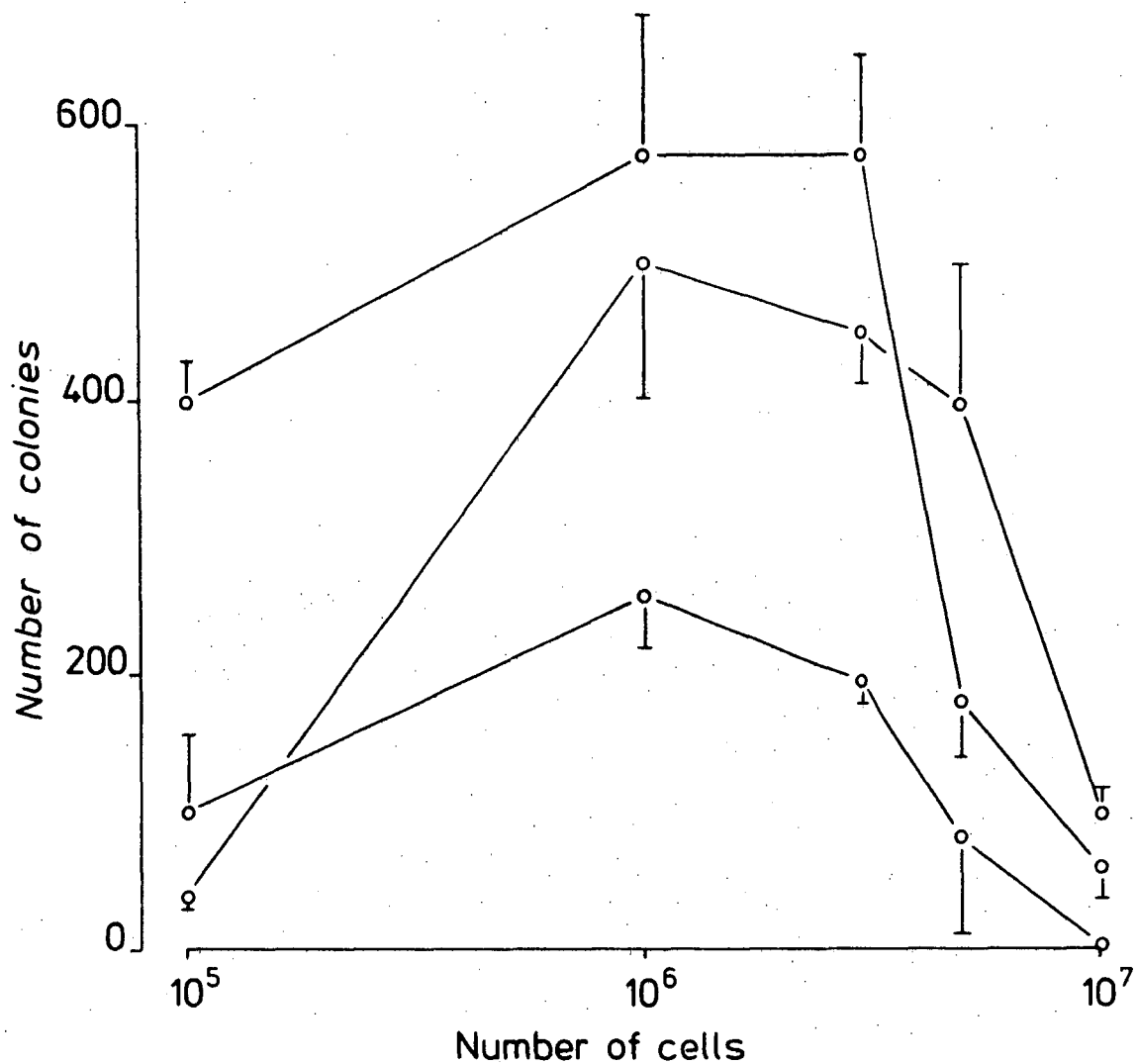


Fig 2.4

The effect of varying cell numbers during the preincubation culture. Mononuclear cells at various concentrations were incubated for 20 h prior to seeding into PHA-containing agar. Each point represents the mean number of colonies from triplicate cultures \pm the standard deviation about the mean when 50 000 cells were seeded into agar. Results from three independent experiments are shown.

2.3.4 Length of agar culture

There was evidence of cell division occurring 2 days after the cells were seeded into agar. From days 2 - 5 intensive cell multiplication was evident with a large number of clusters present. Colonies appeared at approximately day 4, reached maximum numbers at around days 6 - 8 then, after approximately 10 days, most colonies began to degenerate, although occasionally large colonies continued proliferating until day 14. The number of colonies reached a plateau at approximately day 7 (Fig 2.5); this time-span was chosen for future cultures. Cells were therefore cultured in liquid phase for 20 h and in an agar phase for 7 days.

2.3.5 Agar concentration

Fig 2.6 is a representative experiment showing colony formation when agar concentration was varied from 0.3% to 0.75%. At the concentration of 0.3% cell mobility was extremely high resulting in a blanket of cells rendering a satisfactory count impossible. When the concentration was increased to 0.4% the colonies remained disperse and accurate colony counts were still difficult. This is reflected by the large standard deviation shown in Fig 2.6. At the concentration of 0.45% most colonies were relatively disperse, counting was possible although some cultures produced many extremely disperse colonies. On these occasions the scoring of colonies was cumbersome. This was overcome with 0.5% agar which reduced the "spread" of the colonies without affecting their total number. Agar concentrations of 0.6% and above significantly reduced colony growth with regard to both size and number. It is interesting to note that some form of cell growth and mobility was observed with concentrations as high as 0.75%. As the counting of colonies was a tedious and subjective procedure the final

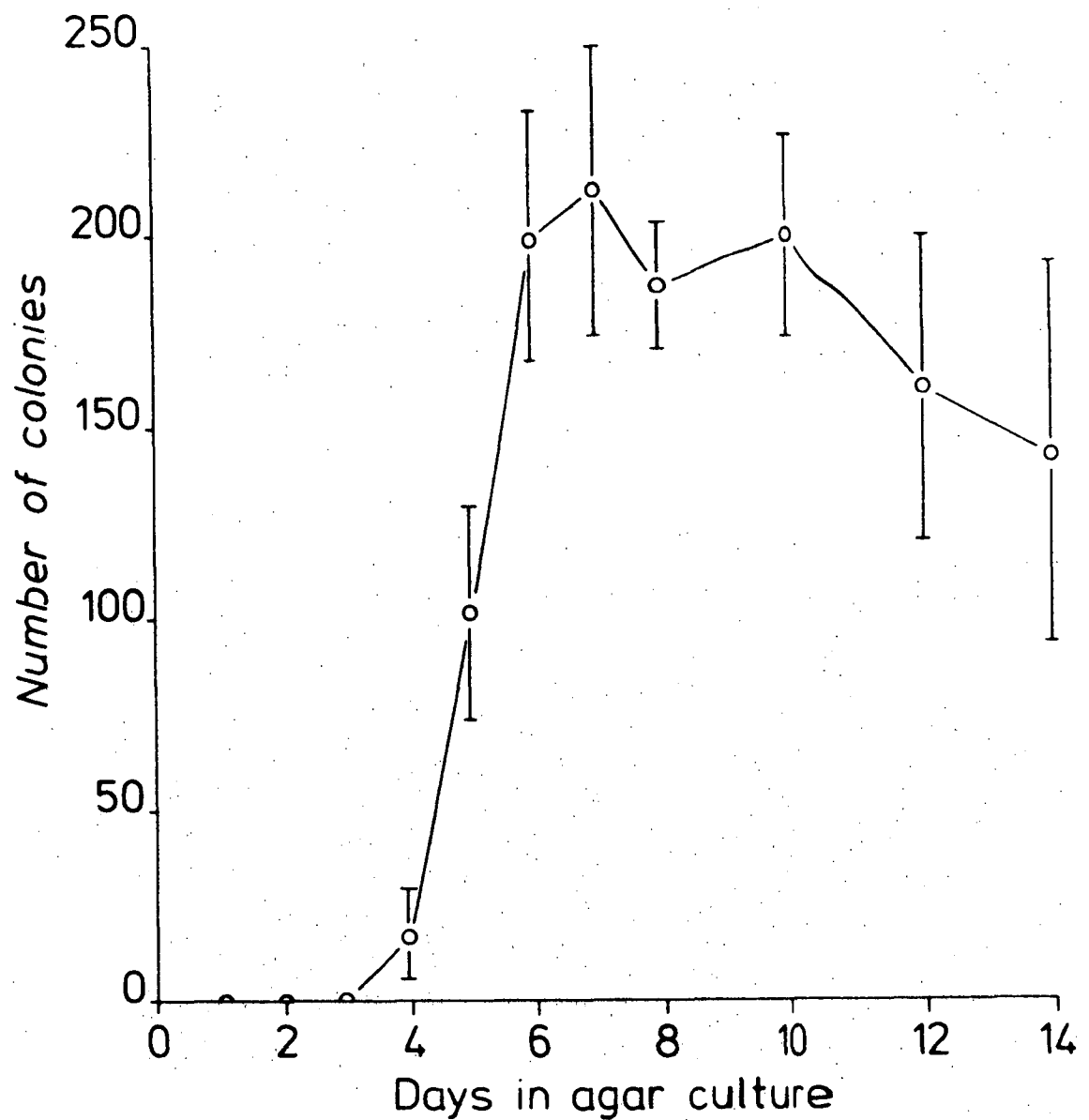


Fig 2.5

Growth kinetics of colony formation. Mononuclear cells were incubated with PHA for 20 h prior to seeding into agar medium. The agar cultures were then incubated for 2-14 days and, because acetic acid was used to lyse the red cells, different plates had to be sequentially examined on different days. Each point represents the mean number of colonies from triplicate cultures \pm the standard deviation about the mean when 50 000 cells were seeded into agar. Results from a representative experiment are shown.

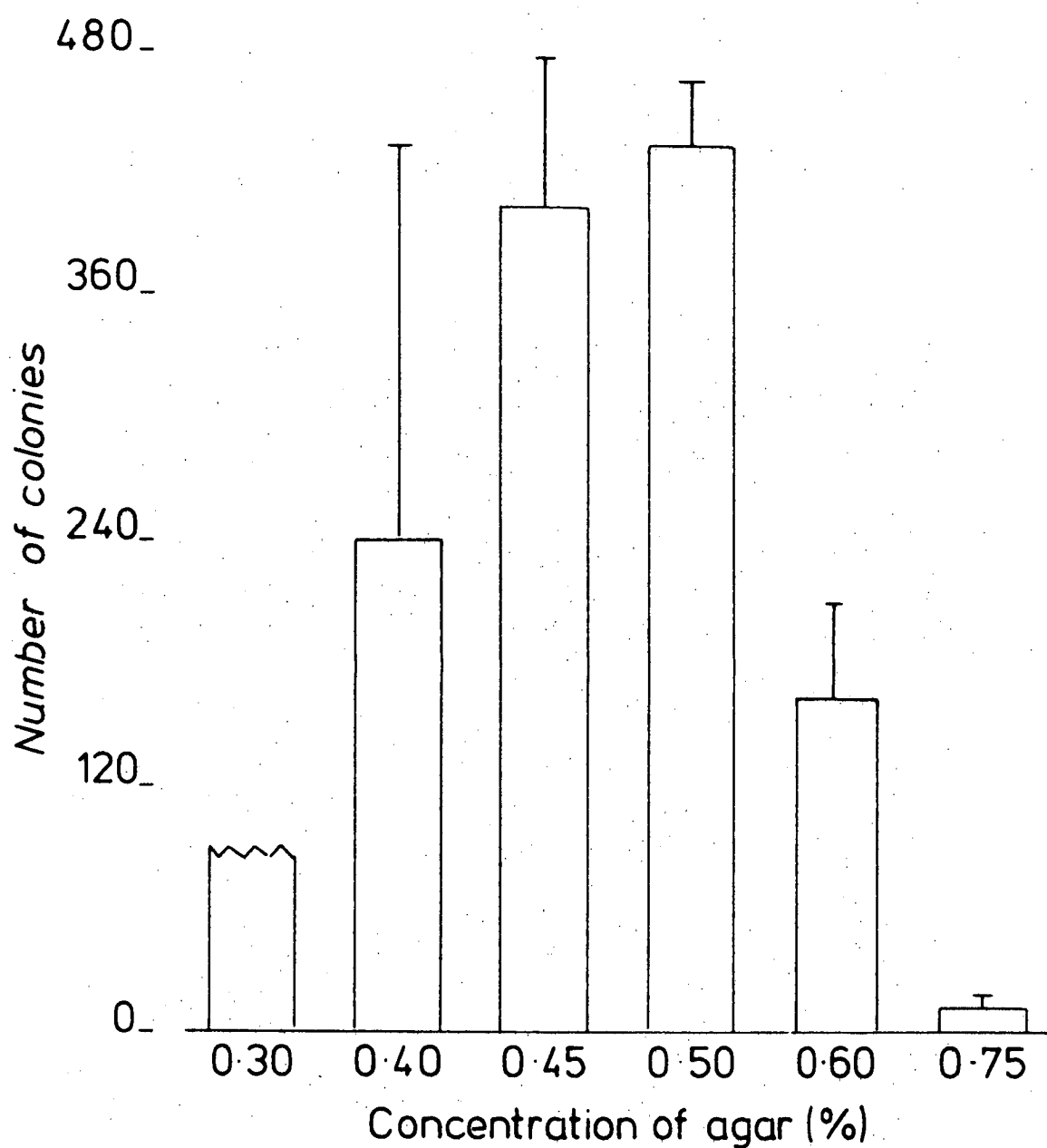


Fig 2.6

The effect of agar concentration on colony growth. Each column represents the mean number of colonies from triplicate cultures \pm the standard deviation about the mean when 50 000 PHA-treated cells were seeded into agar. Note that it was impossible to accurately count colonies with 0.3% agar. A single representative experiment is shown.

concentration of 0.5% was chosen. Although the colonies were slightly smaller than those produced in 0.45% agar they were more confidently scored with a higher degree of accuracy and reproducibility.

In addition to concentration of agar its temperature was also a critical factor. In preliminary experiments a stock of molten agar was held at temperatures ranging from 37°C to 50°C. It was found that if the agar was either a) held at temperatures below 38°C or b) added to cold medium, premature or incomplete gelling occurred. Premature gelling prevented the mixture from being poured onto the petri dishes. Incomplete gelling caused fragments of solid agar to be found floating in a "sea" of liquid, the cells would sink to the bottom of the plate and colony formation was drastically reduced. At the other extreme high agar temperatures either a) caused failure of the cultures to gel or b) destroyed the cells' replicative ability. For all future cultures the stock of molten agar was routinely kept between 40 & 42°C and was always added to warm (or room temperature) medium. Within 30 min of the cultures being prepared they were placed into the incubator.

2.3.6 Concentration of SRBC

An absolute requirement for SRBC during the agar culture can be deduced from Fig 2.7. Concentrations of 1.0% or higher supported the proliferation of the largest number of colonies. As cultures containing more than 1.0% SRBC were difficult to score due to an incomplete lysis of red cells all future cultures were performed with 1.0% SRBC.

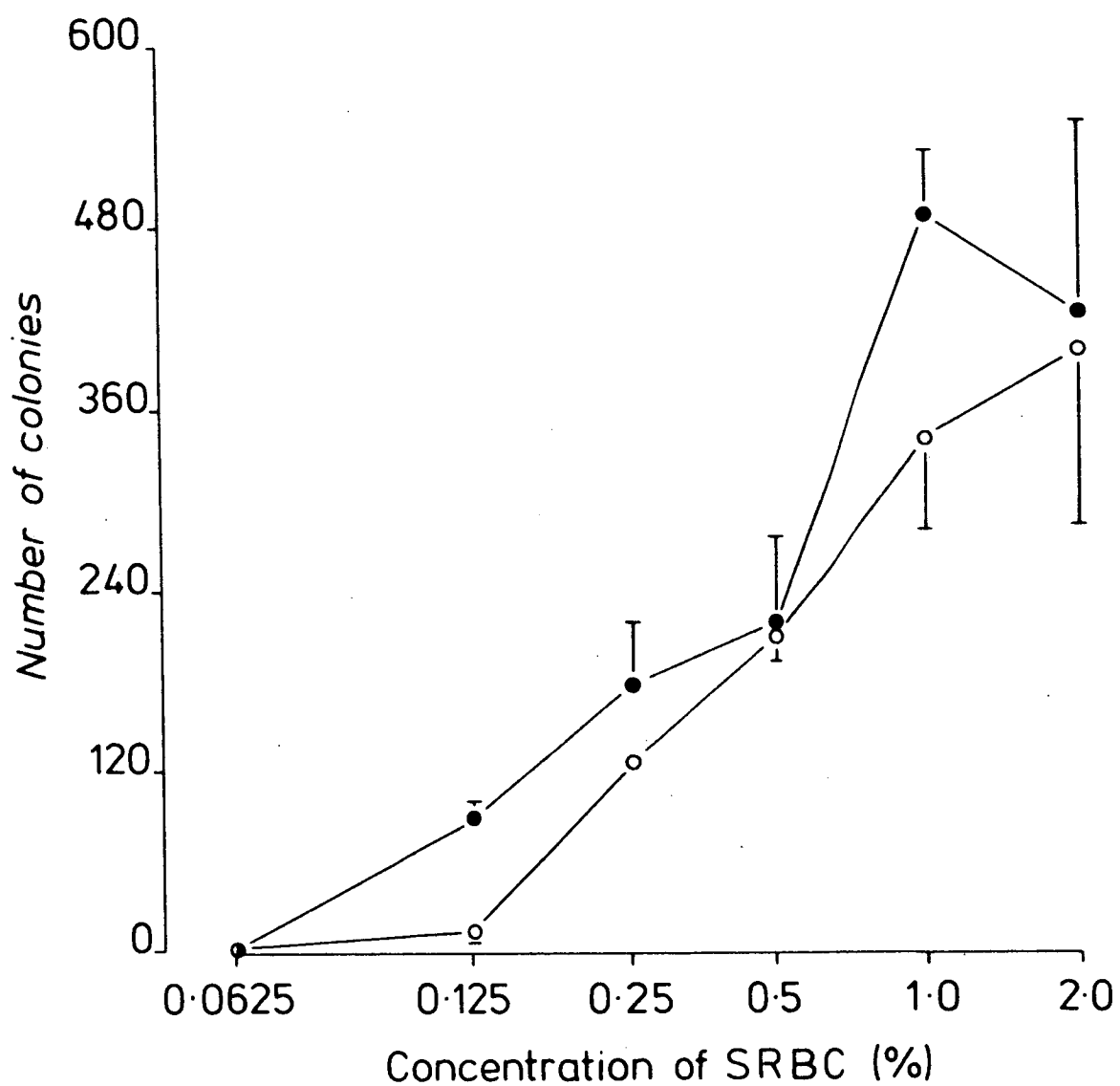


Fig 2.7

The dependence of colony formation on the presence of SRBC. Each point represents the mean number of colonies from triplicate cultures \pm the standard deviation about the mean when 50 000 cells were seeded into agar. Two representative experiments are shown.

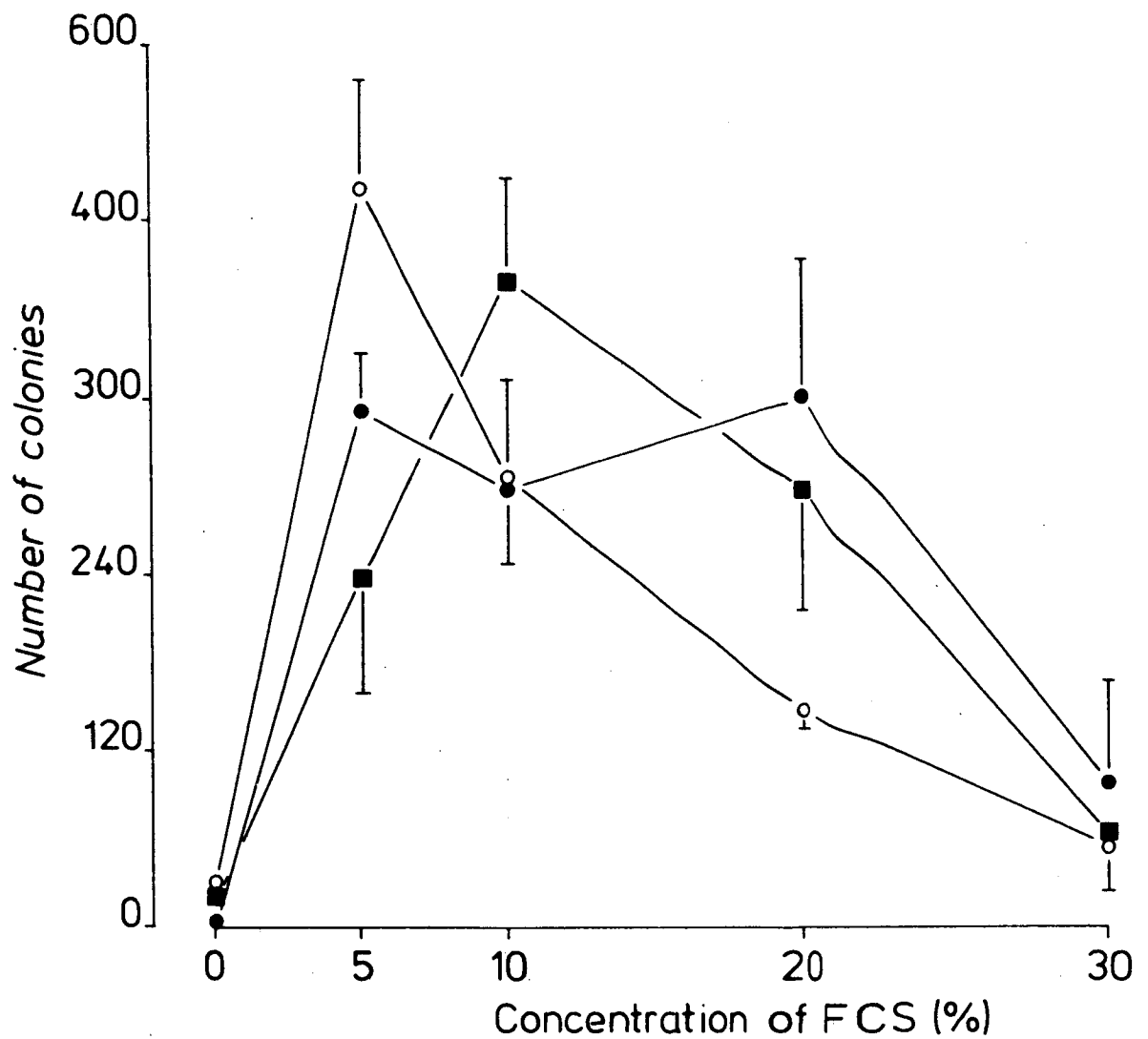


Fig 2.8

The effect of different batches and various concentrations of FCS on colony formation. Each point represents the mean number of colonies from triplicate cultures \pm the standard deviation about the mean when 50 000 cells were seeded into agar. Three representative experiments using three different batches of FCS are shown.

2.3.7 Concentration of FCS

As shown in Fig 2.8 the optimum concentration of FCS showed a slight variation when different batches were used. It was therefore important to titrate all new batches of FCS prior to culturing experiments. The optimal concentration was usually 5%.

2.4 DISCUSSION

In this section a two step (indirect) procedure, based on the method of Claesson et al. (1977a), was described for the production of human T lymphocyte colonies. The technique required a liquid preincubation with PHA, followed by the seeding of these PHA-treated cells into agar, which also contained PHA. Vigorous vortexing and extensive washing of the PHA-treated cells overcame the leucoagglutinating problems caused by PHA. Although the vortexing may have caused a slight reduction in viability the benefit of satisfactory single cell suspensions outweighed this disadvantage. Prior to seeding into agar medium they were again vortexed to prevent reaggregation.

To determine the optimum conditions for colony production many aspects of the technique were varied. A plentiful supply of PHA was required during the preincubation culture. As shown in Fig 2.1 the minimum PHA concentration required was within the range of 10 - 20 ug/ml whereas concentrations as high as 100 ug/ml still allowed colony production to proceed. The concentration of PHA was more critical during the agar culture as the optimum concentration was restricted to a narrow range (20 - 50 ug/ml; Fig 2.2). Due to the length of the culture it was possible that a toxicity of PHA at the higher concentrations was being demonstrated.

After obtaining the optimum concentration for PHA the necessity of the 18 h preincubation was examined by exposing the lymphocytes to PHA for periods ranging from 1 to 48 h prior to seeding into PHA-containing agar. As shown in Fig 2.3 a minimum of 15 h was required to "drive" the colony forming cells to a condition that allowed them to divide when they were seeded into semisolid medium. When the culture period was increased beyond 15 h there was no real improvement in colony formation. This is in agreement with Rozenszajn et al. (1976) who found that a preincubation of 12 h provided adequate stimulation of the colony potentiating cells. As discussed in later sections of this thesis, the necessity for the length this preincubation culture was related to the activation and function of cells that were accessory to the proliferating cells rather than directly to the colony forming cells.

The appearance of colonies followed 4 recognisable phases. Very little cell division was seen during the first 1-2 days after the PHA-treated cells were seeded into the agar as the cells were probably undergoing the premitotic stages (e.g. G₁, S, G₂ phases) of cell mitosis and readjusting from a liquid to semisolid medium. After 2 days colony formation was rapid until about day 7 when the number of colonies reached a peak value. After this time colony numbers remained constant and from day 10 onwards they began to degenerate, probably due to a lack of stimulation and growth factors in the medium. On occasions a few large colonies continued growing until at least day 14 but most colonies had a limited lifespan under these conditions.

During the preincubation culture the concentration of mononuclear cells was important. As shown in Fig 2.4 high cell concentrations did not allow adequate stimulation as evidenced by the

failure of these cells to form colonies. At high cell concentrations the effects of suppressor cells may override the positive attributes of other cell types. In a similar study Claesson et al., (1977a) found that a minimum of 50 000 cells was required during the preincubation culture to enable proliferation when they were seeded into agar. For the experiments described herein the lowest cell concentration (1×10^6 /ml) usually allowed colony growth, however, as will be discussed in section 3, there was a minimum number of cells required during the agar culture. The demonstration of a cell concentration threshold, below which colonies do not develop, is good evidence that colony cells require "helper-factors" from other cell types.

The production of adequate numbers of colonies was also dependent on the presence of FCS throughout both culture steps and a source of SRBC during the agar culture. As shown in Fig 2.8 the optimum concentration of FCS varied with different batches and it was therefore essential to titrate all new batches of FCS. Colony growth was dose dependent on SRBC and, as shown in Fig 2.7 a final concentration of 1% was optimal. The dose response nature would suggest that the SRBC were actively involved in T colony formation.

In many of the earlier reports the concentration of agar was 0.3% (Table 1.1). In the present work the agar concentration of 0.5% was found necessary to restrict the freely mobile proliferating cells. Lower agar concentrations produced disperse colonies that overlapped causing colony identification difficult whereas higher concentrations were found to be inhibitory (Fig 2.6). It is interesting to note that with the low agar concentrations used by other investigators many colonies developed on the surface of the agar (Fibach, Gerassi and Sachs, 1976; Rozenszajn et al., 1978). The

frequency and morphology of different forms of colonies is discussed in section 3.

2.5 SUMMARY

A two step procedure was developed for the culturing of human lymphocytes in semisolid agar. Mononuclear cells were separated from peripheral blood and incubated overnight with PHA in a liquid phase. Colonies of 50 or more cells developed when these PHA-treated cells were seeded into agar medium containing PHA and cultured for a further 7 days. Essential for this technique was a continued supply of PHA, a source of SRBC during the agar culture and a high agar concentration to restrict the freely mobile proliferating cells. The two step nature and a sufficient plating efficiency makes this system suitable for critical studies of cell mediated proliferative responses.

S E C T I O N T H R E E

A N A L Y S I S O F C O L O N Y F O R M A T I O N

3.1 INTRODUCTION

In the previous section the optimum conditions required for the growth of human T lymphocyte colonies were established. Further analyses of colony growth characteristics were performed and are reported in this section of the thesis. The T cell nature of cells within the colonies was examined through their ability to spontaneously rosette with sheep red blood cells while the T cell nature of the colony progenitor cells was investigated with E-rosette depletion experiments. Colony formation was examined by scoring according to size, shape and location of growth (i.e. within the agar matrix or on the surface of the agar). The relationship between the number of cells seeded and the number of colonies produced was determined through cell dose experiments and the variability of colony numbers produced by different individuals was investigated.

3.2 METHODS

3.2.1 Harvesting individual colonies from the agar

Cells were harvested after the red cells had been lysed with warm tris-NH₄Cl. Single colonies were removed from the agar matrix with the aid of a shortened Pasteur pipette which had been specifically prepared to comprise a fine narrow tip, approximately 2 cm in length with an internal diameter of 0.5 to 1.0 mm. A complete circle was "cut" around the colony to be isolated by disrupting the agar with the tip of a 26 G needle. This enabled easier removal of the colony from the gelatinous agar-medium. To remove the colony a minute volume of medium was added over the surface of the culture plate and the pipette was introduced into the agar, around the colony, at an

angle of 45° . The colony was held at the end of the narrow pipette by allowing slight suction, taking care that it was not sucked too far up the pipette. The colony was then removed and added to a well of a sterile microtitre tray or a small test tube containing a small volume of medium. Disruption of cells out of the agar plug was performed by aspirating these "plugs" through a 26 G needle at least 10 times and the suspension was incubated overnight at 37°C . For colonies that grew on the surface of the agar 0.5 ml medium was added to the agar. These "surface" colonies floated and were easily harvested. They were vortexed and washed to obtain single cell suspensions and incubated overnight at 37°C .

3.2.2 Drying and staining of agar cultures

3.2.2.1 Preparation of gelatinised slides

Large (75 x 50 mm) glass slides, cleaned with chromic acid, were dipped into a gelatinizing solution containing 0.5 g gelatin, 1 ml 5% potassium dichromate ($\text{K}_2\text{Cr}_2\text{O}_7$) made up to 100 ml with distilled water. The slides were dried at 56°C and stored in a cool dry place.

3.2.2.2 Removal and drying of agar cultures

Entire agar cultures were removed from the petri dishes by first detaching the agar from the edge of the petri dish by running a spatula around the inside of the culture dish (i.e. between the edge of the agar and the inside edge of the dish). The plate was then submerged in a larger petri dish containing fresh distilled water. By rocking the submerged culture dish the agar usually floated to the surface. For difficult cases the agar was floated by gently lifting around the edges of the agar with a spatula. Once the agar floated a

clean gelatinised slide (75 x 50 mm) was placed below it. The agar and slide were carefully extracted, excess water around the agar was removed and the wet-agar-slide preparation was allowed to dry by incubating overnight at 37°C.

3.2.2.3 Staining of dried agar cultures

Dried agar preparations were placed in a plastic tray containing moistened paper for humidification purposes. The dried agar was completely covered with an aceto-orcein stain prepared as a stock solution containing 0.3 g orcein, 30 ml acetic acid and 20 ml double distilled water. Care was taken to prevent the stain from entering between the slide and the agar as this caused "bubbles" to appear in the agar. The plastic tray was covered to maintain humidity and the slides were incubated at room temperature for 30 min after which time the excess aceto-orcein was washed away and the stained slides were allowed to dry at 60°C for 30-60 min. These slides were mounted with DePeX mounting medium under large (75 x 50 mm) coverslips and stored in the dark where they remained stable for a number of years.

3.2.3 Surface marker analysis of the colony cells

Tests for E-rosettes

E-rosette forming cells (E-rosettes) were detected after a 10 min, 37°C incubation of SRBC with either cells from a single colony or pooled colony cells. E-rosettes of cells obtained from a single colony were performed by adding 10 ul of 1% SRBC to a well of a microtitre tray containing the cells, whereas E-rosettes of pooled colony cells were performed in tubes containing the SRBC and colony cells at an approximate ratio of 10:1. Following incubation the

suspensions were gently centrifuged and incubated overnight at 4°C. Counting of the rosettes was performed under light microscopy after resuspension with rosette counting fluid containing crystal violet and glutaraldehyde. A rosette was defined as a lymphocyte surrounded by 3 or more adhered SRBC.

Tests for M-rosettes

Mouse red cell rosettes (M-rosettes) were tested for in an identical way to E-rosettes with the single exception being that mouse red cells were substituted for the SRBC. M-rosettes were performed because they detect a subpopulation of B lymphocytes (Catovsky et al., 1976) and they would therefore serve as a control for any non-specific "sticking" of red cells and colony lymphocytes that may be caused by the presence of agar.

Tests for surface immunoglobulin

Surface membrane immunoglobulin bearing cells (SmIg) were identified by a direct immunofluorescent technique using polyvalent fluorescein conjugated goat anti human-Ig. Pooled colony cells were incubated with this fluorescent antibody for 30 min at 4°C, washed twice, then mounted under coverslips with veronal buffered glycerol and examined by fluorescent microscopy (Nairn, 1976).

3.2.4 Cell dose response curves

To determine the relationship between the number of seeded cells and colonies, graphs of colony counts versus cells seeded were performed. The PHA-treated cells were seeded into agar at concentrations within the range of $5 - 100 \times 10^3$ /ml and experiments were performed in triplicate. The graphs were drawn by plotting the individual colony counts rather than the mean and standard deviation.

Computer defined lines of best fit and their correlation coefficients were calculated by considering all the points (excluding the origin) rather than their mean values.

3.2.5 E-rosette depletion

Mononuclear cells (1.0 ml at 5×10^6 /ml) were mixed with equal volumes of 2% SRBC in 10 ml sterile polystyrene plastic tubes. The preparations were incubated for 10 min at 37°C, then at 4°C for 60 min after which time the tubes were gently shaken to resuspend the cells that had settled to the bottom of the tube. Approximately 3 ml Histopaque (Isopaque-Ficoll) was "layered" under the cell suspensions which were then centrifuged at 400 x g for 40 min at 4°C. Both the mononuclear band (non-E-rosetted cells) and the red cell pellet (E-rosetted cells) were harvested. The mononuclear band was washed prior to culturing in the two step colony assay. The pelleted red cells were vigorously vortexed to dislodge the bound SRBC from the mononuclear cells, then to lyse the red cells 0.9 ml sterile distilled water was added to the pellet. The mixture was vortexed and isotonic rescue was performed by adding 0.1 ml 10X- concentrated PBS. The rosetted cells were washed 3 times prior to culturing in the two step colony assay. Subsequently unseparated mononuclear cells, E-rosette enriched and E-rosette depleted cells were assayed for their colony forming ability. E-rosette analysis was performed on each of these preparations and 50 000 cells (PHA-treated) were seeded into agar.

3.2.6 Variation of colony formation

The number of colonies from different individuals was compared when the cultures were performed in parallel.

The number of colonies from the same individual was

compared on different days over either a short time span (1 week) or a longer time span (approximately 3 months).

3.3 RESULTS

3.3.1 Surface marker analysis of colony cells

Surface markers were analysed on either cells obtained from single colonies plucked from the agar or on pooled colony cells obtained from the surface of the agar. Cells from the surface of the agar were easily harvested and maintained a high viability. The method required to harvest colony cells from within the agar was harsh on the cells and consequently the viability was low. Tables 3.1 to 3.3 show a close relationship between the proportion of viable cells and those forming E-rosettes for both the single and pooled colony data. Results from Table 3.1 show that all colonies contained cells that were positive for E-rosettes.

The percentage of cells forming mouse-rosettes was low for single colony cells (Table 3.4) demonstrating that the majority of cells did not belong to the subpopulation of B lymphocytes that bind mouse red cells (Catovsky et al., 1976). More importantly the cells cultured in agar did not become "sticky" and attach, non-specifically, to red cells.

The presence of some cells positive for SmIg was shown in Table 3.3. However the proportion of E-rosette forming cells and SmIg binding cells accounted for more than 100% of the cells indicating that non-specific fluorescence may have occurred.

TABLE 3.1

E-rosette forming cells from single colonies

	Number of E-rosette forming cells	Number of cells per colony	Percent E-rosette forming cells
	25	61	41
	19	45	42
	39	66	59
	31	42	73
	20	70	29
	31	62	50
	42	68	62
	21	39	54
	26	37	70
	35	53	66
	38	61	62
	98	164	60
	62	127	49
	21	48	44
	50	98	51
	113	160	71
	249	314	79
	51	78	65
	14	33	42
	31	47	66
	85	110	77
	11	37	30
	20	51	39
	29	47	62
	32	54	59
	16	37	43
	29	92	32
	37	65	57
	12	30	40
	71	102	70
TOTAL	1358	2298	60

TABLE 3.2

Viabilities of cells from single colonies

	Number of viable cells	Number of cells per colony	Percent viable cells
	21	39	54
	26	37	70
	35	53	66
	38	61	62
	98	164	60
	62	127	45
	21	48	44
	50	98	51
	113	160	71
	25	31	81
	224	283	80
	51	78	65
	14	33	42
	31	47	66
	85	110	77
	27	63	43
	31	61	51
	12	43	28
	78	109	72
	52	60	87
TOTAL	1094	1705	64

TABLE 3.3

Surface marker analysis and viabilities of pooled (monolayer) colony cells

EXP.NO	E-rosettes			Viability			SmIg fluorescence		
	+ve	total	% +ve	viable	total	% viable	+ve	total	% +ve
I	364	488	75	353	509	69	N.D.		
II	96	133	72	93	112	83	N.D.		
III	487	495	98	215	223	96	N.D.		
IV	428	466	92	98	103	95	50	186	27
TOTAL	1375	1582	87	759	947	80	N.A.		

N.D. not done

N.A. not applicable

TOTAL percentages were calculated by pooling all the results together rather than averaging the percentages obtained for each individual experiment

Note the total percentage for surface markers for EXP.NO IV exceeded 100%

TABLE 3.4

M-rosette forming cells from single colonies

Number of M-rosette forming cells	Number of cells per colony	Percent M-rosette forming cells
0	25	0
0	34	0
7	52	13
4	21	19
0	63	0
0	43	0
15	97	15
0	41	0
0	21	0
8	61	13
TOTAL 34	458	7

3.3.2 Morphology of the colonies

Seeded lymphocytes produced colonies either within or on the surface of the agar. Colonies that developed within the agar could be broadly classified as small (containing 50-100 cells), medium (containing 101-500 cells) and large (containing >500 cells). Small colonies were usually compact (Fig 3.1A), medium were usually disperse (Fig 3.1D) and large colonies contained many cells tightly packed in the centre and loosely arranged around the periphery (Fig 3.1C), while colonies developing on the surface of the agar were flat and formed a monolayer (Fig 3.1B). Clusters (10-49 cells) also developed (Fig 3.1E). Individual cells from the colonies appeared lymphoid or lymphoblastoid-like (Fig 3.1F) and did not contain vacuoles. The cellular morphology was somewhat distorted due to the drying and flattening of these colonies.

3.3.3 Cell dose response curves

A linear relationship was obtained when the number of cells plated was plotted against the number of colonies. Four representative curves are shown in Fig 3.2. When these curves were extrapolated to zero cell dose they did not pass through the origin. It can be seen in Fig 3.2 that some points fell outside the linear region. This was occasionally noted at either very high or very low cell concentrations. With an increase in the number of colonies at higher cell concentrations an apparent increase in the size (i.e. number of cells per colony) of the colonies was noted.

Fig 3.1

Photographs of human T lymphocyte colonies in agar stained by aceto-orcein.

(A) small compact colony growing within the agar
(magnification x 80)

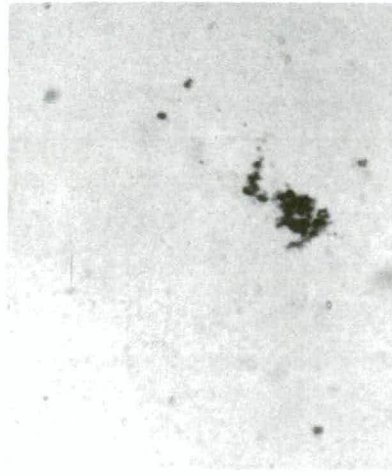
(B) monolayer colony growing on the surface of the agar
(magnification x 80)

(C) large tight centred colony growing within the agar
(magnification x 80)

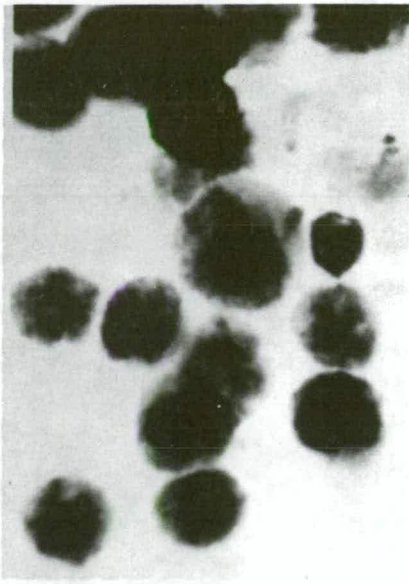
(D) medium disperse colony growing within the agar
(magnification x 80)

(E) cluster (10 - 49 cells) growing within the agar
(magnification x 80)

(F) single cells obtained from a colony growing within the agar. Note the homogeneity of cell morphology and the absence of vacuoles.



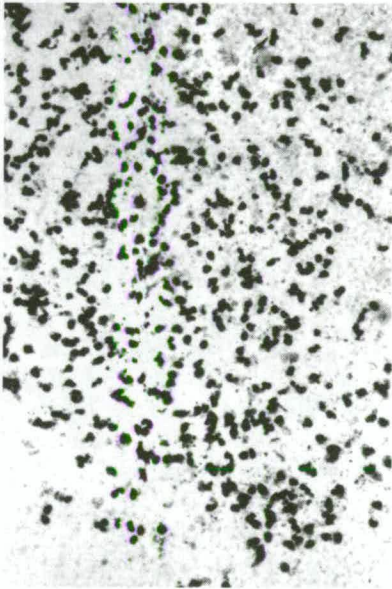
E



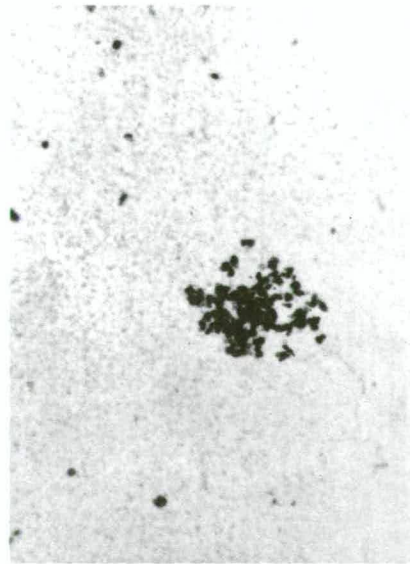
F



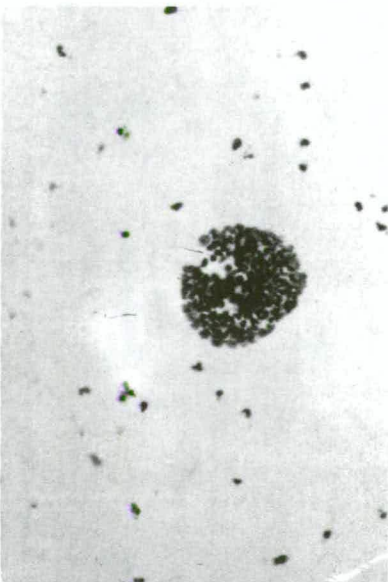
C



D



A



B

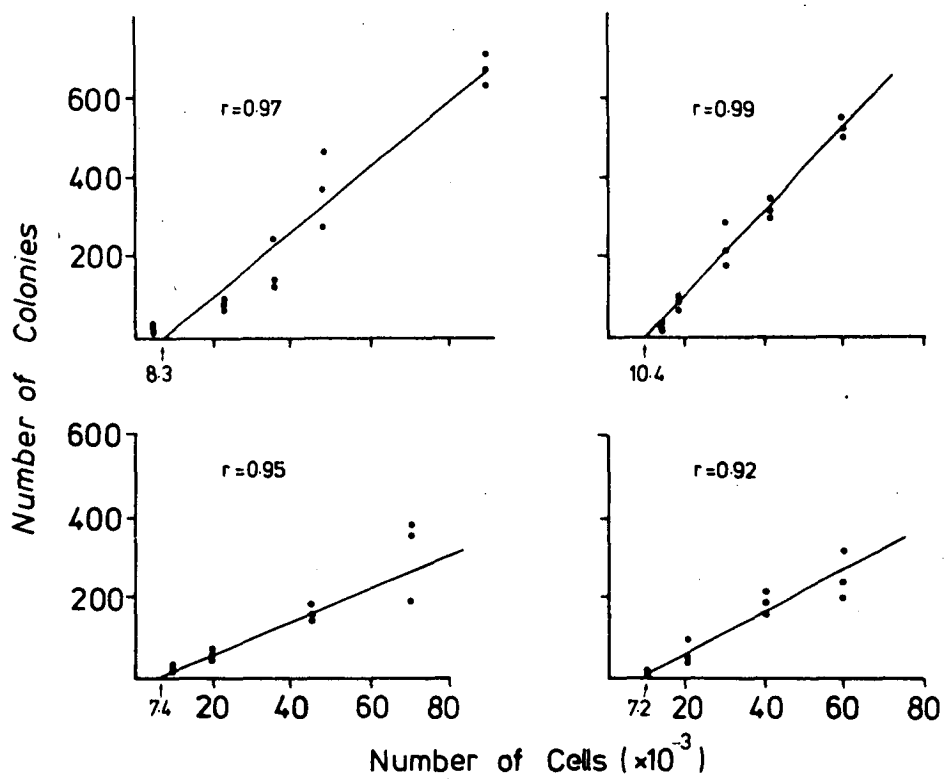


Fig 3.2

Relationship between number of colonies and number of cells seeded. Each point represents the number of colonies produced from each of the triplicate cultures. r = correlation coefficient. Four representative experiments are shown.

3.3.4 Depletion and enrichment of E-rosettes

As shown in Table 3.5 the removal of E-rosette forming cells abolished colony formation whereas the E-enriched fraction and total mononuclear cells produced good colony growth. Generally, colonies developing from the E-enriched preparations were smaller than their non-separated counterparts. It should also be noted that 50 000 cells were cultured irrespective of whether they were total mononuclear cells or E-rosette manipulated and therefore, on the basis of total numbers of E-rosette positive cells plated, the mononuclear cell preparation produced the highest plating efficiency. From Table 3.6 it can be seen that the E-enriched and E-depleted samples were not exclusively E-rosette positive and negative respectively.

3.3.5 Variation of colony growth

The frequency of colonies produced when equivalent numbers of seeded cells from different donors were cultured showed considerable variation when either the total number of colonies or the types of colonies (i.e. small, medium etc) was compared (Table 3.7). This variation in total colony formation was also observed when the cells from the same donor were cultured either on consecutive days (Fig 3.3) or over a longer time-span (* of Table 3.7) or the cells from different donors were cultured at the same time (Fig 3.4). This variation could not be explained by different numbers of T lymphocytes plated. On occasions, as is shown in Table 3.7, cells from individual donors failed to produce satisfactory colony growth.

Table 3.5

Colony growth from E-rosette manipulated cells

cells seeded	Number of colonies		
	Exp I	Exp II	Exp III
unseparated mononuclear	240 \pm 20	200 \pm 16	426 \pm 37
E-rosette enriched	171 \pm 42	225 \pm 26	392 \pm 71
E-rosette depleted	6 \pm 2	7 \pm 3	12 \pm 7

Results represent mean \pm standard deviation of triplicate cultures from 3 experiments.

The experiment numbers (I, II and III) correspond to the same experiments in Table 3.6

Table 3.6

E-rosette percentages of E-rosette manipulated cells

cells	percent E-rosettes		
	Exp I	Exp II	Exp III
unseparated mononuclear	75	62	38
E-rosette enriched	91	87	80
E-rosette depleted	13	4	2

The values shown were determined by examining at least 200 cells for their E-rosette forming capacity prior to the liquid preincubation culture.

The experiment numbers (I, II and III) correspond to the same experiments in Table 3.5

Table 3.7

Variation of T lymphocyte colonies with different cultures

Number of colonies per 50 000 cells seeded					
EXP.NO	Large	Medium	Small	Monolayer	TOTAL+
*I	0	104 \pm 21	19 \pm 18	0	123
*II	28 \pm 12	2 \pm 2	166 \pm 31	0	196
*III	0	360 \pm 100	0	31 \pm 14	391
IV	28 \pm 10	134 \pm 25	0	0	162
V	18 \pm 13	0	445 \pm 106	48 \pm 23	511
VI	0	174 \pm 32	0	0	174
VII	0	0	589 \pm 123	0	589
VIII	0	1 \pm 1	17 \pm 10	0	18

Values are mean \pm standard deviation for triplicate cultures

* Cultures from the same donor on different days over a 3 month period

+ Total equals the sum of the mean number of colonies for the different colony types

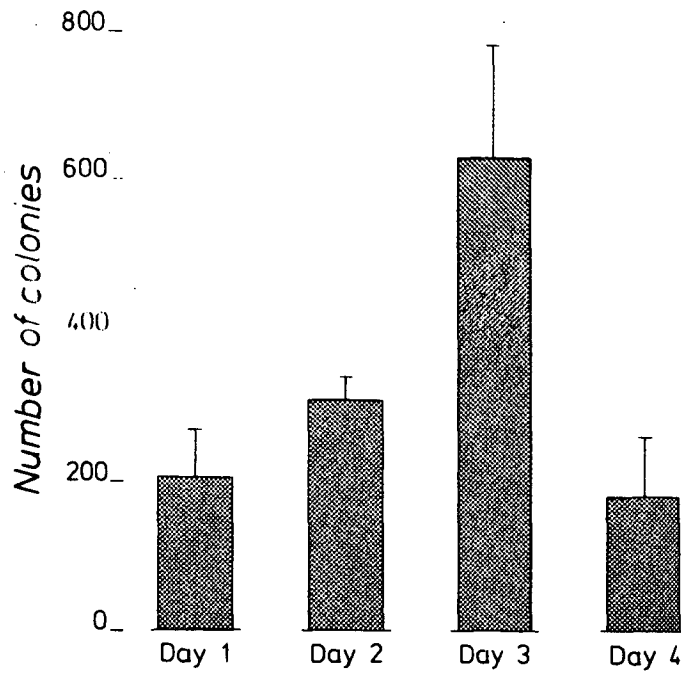


Fig 3.3

Variation of colony formation when cells from a single donor were cultured on four consecutive days. Columns represent the mean of triplicate cultures when 50 000 cells were seeded. Vertical bars indicate the standard deviation of the mean.

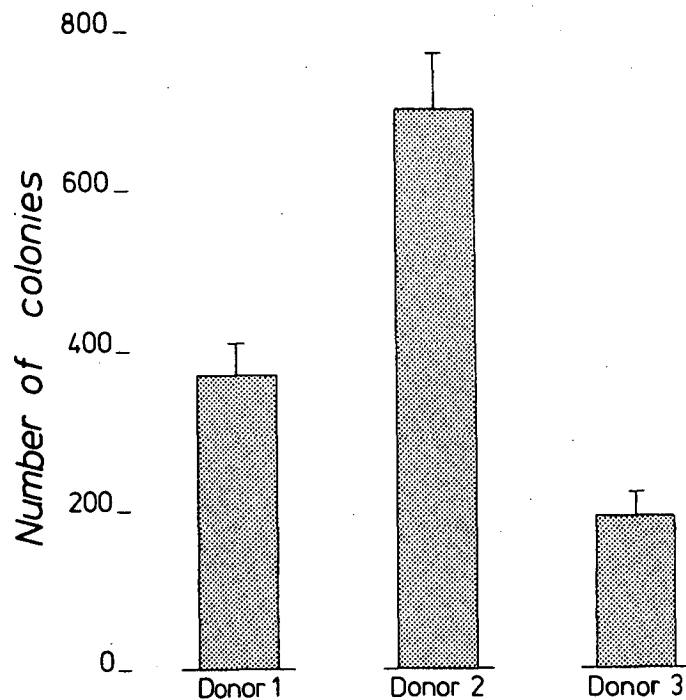


Fig 3.4

Variation of colony formation when cells from three donors were cultured in parallel. Columns represent the mean of triplicate cultures when 50 000 cells were seeded. Vertical bars indicate the standard deviation of the mean.

3.4 DISCUSSION

The capacity of the colony cells to form E-rosettes demonstrated a T cell lineage of these cells. This T cell nature has been substantiated in a number of independent reports through the cells' ability to react with SRBC or polyvalent anti-T cell serum and not react with polyvalent anti-Ig (section 1). Non-viable lymphocytes lose their ability to bind SRBC (Coombs et al., 1970) and, as shown in Tables 3.1, 3.2 & 3.3, a direct correlation between viability and the proportion of cells forming E-rosettes was observed. This suggests that the viable cells bound SRBC and could therefore be classified as T cells. Furthermore, every single colony assayed possessed cells that formed E-rosettes indicating that all of the colonies contained T lymphocytes. The attachment of SRBC to colony cells was not due to the agar causing them to become "sticky" because most of the cells did not bind to mouse red cells (Table 3.4). However a small proportion did bind, suggesting that some of the colonies contained B cells. In addition a fraction of pooled colony cells reacted with the FITC-conjugated anti-Ig antibody but this value may have been excessive because the sum of E-rosette forming and SmIg binding cells accounted for more than the total number of cells present (Table 3.3). As activated T lymphocytes express receptors for the Fc fragment of immunoglobulins (Parish, 1975) it is highly probable that the Fc portion of the polyclonal antibody non-specifically attached to the T cells through these receptors. Although non-specific binding may account for a significant part of this activity it is still possible that some cells within the colonies were B cells as reflected by the small proportion of colony cells that bound mouse red cells (Table 3.4). It is therefore likely that although the colonies were

comprised of mostly T cells, some non-T cells were present, albeit in minor proportions. A similar finding has been reported by Farcet et al. (1980b). All colonies cannot therefore be regarded as clones of T lymphocytes.

Further evidence to support the T cell nature of the colonies can be derived from Table 3.5 which shows that E-rosette depleted cells did not form colonies whereas E-rosette enriched cells produced good colony growth. Such a finding has significant support from many investigators thereby providing strong evidence that the colony progenitor cell (TLCFC) is a mature T lymphocyte rather than an immature progenitor cell. Colonies produced from the E-rosette enriched cells were not as large as those produced from the unseparated cells. Furthermore, although total colony numbers were similar, in terms of colonies produced per T lymphocytes plated the E-enriched preparation had a lower plating efficiency than the unseparated mononuclear cells. This may seem to be an incongruous finding, as one would anticipate that because the E-enriched preparation contained more T lymphocytes it should produce more colonies. A possible explanation, pre-empting the evidence for cellular cooperation discussed in sections 5 & 6, is that during the rosetting procedure "helper cells" were lost and colony growth was restricted due to an inadequate supply of "helper" factors.

The number of colonies grown was directly proportional to the number of cells plated (Fig 3.2). Claesson et al. (1977a) demonstrated a direct relationship using a similar system and comparable results with other culture systems have been reported (Rozenszajn et al., 1975; Fibach, Gerassi and Sachs, 1976; Mercola and Cline, 1979; Alder, Morley and Seshadri, 1982). In contrast,

non-linear relationships have also been described (Farcet, Kincade and Moore, 1980a; Spitzer et al., 1980). The apparent simple direct relationship between cells and colonies may therefore be more complicated than the linearity of the curve predicts. As shown in Fig 3.2 the linear curves, when extrapolated to zero colonies do not intersect the X-axis at the origin. At high colony numbers the size of the colonies (i.e. number of cells per colony) was larger than at low colony numbers. It is therefore likely that colony development involves more than a mere proliferation of the colony forming cells. Evidence for this may also be obtained from the culture experiments where a concentration threshold occurred, below which no colony growth was observed (Rozenszajn et al., 1975; Fibach et al., 1976; Gerassi and Sachs, 1976; Riou et al., 1976; Wilson and Dalton, 1976; Shen et al., 1977; Ulmer and Flad, 1979). This suggests that a build up of accessory cells may be required to produce sufficient "helper" activity to promote colony formation. Although Claesson et al. (1977a) found that a liquid preincubation abolished the need for minimum cells to be seeded they also found that no colonies developed when less than 50 000 cells were incubated during the preincubation culture suggesting that cellular interactions were important at the early stages. These findings and the observations within this section that linear cell dose response curves do not pass through the origin and that at higher colony frequencies colony sizes appeared to be larger provide further evidence that cellular interactions are important for colony development.

The colonies that developed within this system were broadly classified according to their size and location of growth. Small, medium and large colonies grew within the agar matrix while monolayer

colonies developed on the surface of the agar (Fig 3.1). The individual cells within these colonies appeared to be lymphoid- or lymphoblastoid- like and did not contain vacuoles. However, as the morphology was somewhat disturbed through the drying procedure it was difficult to accurately describe them. Colony production was also accompanied by the growth of smaller cell groups, or clusters. These clusters contained 10-49 cells and were usually compact and difficult to distinguish against a background of many cells. It is unlikely that clusters and colonies represent different T cell populations but merely reflect the conditions in which the culture was performed. (A consideration of these clusters is discussed in later sections of this thesis.) Fibach et al. (1976) defined colonies growing within the agar as type I and colonies growing on top of the agar as type II. By comparing the two systems it would appear that the small, medium and large colonies could be categorized as type I whereas the monolayer colonies would belong to the type II category. Type II colonies have been found to be more prolific than type I colonies (Fibach et al., 1976; Zeevi, Goldman and Rozenszajn, 1977; Rozenszajn et al., 1981) and it has been suggested that they belong to different functional populations of T lymphocytes (Rozenszajn et al., 1981). With the present work and as seen from Table 3.7 this frequency ratio was reversed; the monolayer (type II like) was the uncommon type. An explanation for this difference might be the concentration of the agar. At low agar concentrations, such as 0.32% used by Rozenszajn et al. (1981), cell movement would be excessive and many cells could migrate to the surface of the agar where they aggregate to form flattened colonies. The 0.5% agar used in the present experiments would prevent this excessive cell movement.

The frequency of colony formation varied with different cultures. The variation could not be attributed to any single colony type. It appeared that one particular colony type would dominate different cultures suggesting the conditions were partly responsible for the different sizes and shapes of these colonies. This possibility and the difficulty in categorising some colonies, that were intermediate between the classifications discussed, suggested that total colony counts would be a more reproducible parameter than that of the colony types. For these reasons, only total colonies will be considered in the remainder of this thesis.

The wide range of total colony frequencies between different individuals (Table 3.7) was not due to the culture conditions because a similar variation was found when three individuals were cultured in parallel (Fig 3.4). When a single individual was cultured on consecutive days a similar range was again found (Fig 3.3). The variation was not due to the culture technique because replicate cultures were consistently within a reasonable range, nor was the variation due to the absolute number of T cells cultured as they were similar on all occasions. The reasons for such variations may lie in a number of in vivo and in vitro phenomena including immune statuses of donors, biological cycles, monocyte-lymphocyte interactions or differential PHA-responses by lymphocyte subpopulations which vary with different individuals. Normal individuals have been shown to respond to varying degrees with PHA (Fan et al., 1977; Roberts, 1980) while subpopulations of lymphocytes demonstrate differential responses to PHA (Moretta et al., 1976). It has also been shown that PHA-responses undergo a biphasic cycle throughout an entire day with maximum responses occurring at different

times (Eskola et al., 1976). The range for PHA-induced colony formation reported throughout this thesis is similar to those described by other workers (Table 1.1). A lack of colony formation has been seen in a number of reports (Riou et al., 1976; Zeevi et al., 1977; Dao et al., 1978; Klein et al., 1981a) and may be due to suboptimal conditions or the presence of excessive numbers of suppressor cells. Such an absence of colony growth can also be seen in Table 3.7. It is not known whether the failure of colony production on these occasions was due to any underlying clinical disorders. As the cells were obtained from apparently normal volunteers it is possible that the trauma of experimentation rather than true cell defects may have been responsible for this lack of colony production. As discussed in section 6 the lack of colony growth was probably due to accessory cell defects as these cells may require conditions for optimum function different from those required by the proliferating colony cells.

3.5 SUMMARY

Colonies produced with the technique outlined in section 2 were further analysed in this section of the thesis. Their T cell nature was evidenced by the ability of the colony cells to form E-rosettes. The inability of E-rosette depleted cells to develop into colonies compared to the E-rosette enriched cells was supportive of this finding and indicated a T cell origin for the colony forming cells. Although all colonies contained a high proportion of E-rosette positive cells it was evident that some non-T cells were present in a minority of the colonies and, as high cell mobility was also evident it is unlikely that all colonies were true clones of T lymphocytes. A direct relationship between the number of cells seeded and the number

of colonies produced was consistently obtained. The linear curves did not pass through the origin, however, and at high colony numbers the total number of cells per colony was greater than at low colony numbers providing indirect evidence for colony formation requiring some form of "external assistance", possibly from other cell types. The colonies could be loosely categorized according to their size, shape and location of growth, however, a considerable variation was observed with different cultures. In addition there was a marked difference in colony frequencies from the cells of different donors cultured at the same time or cells from the same donor cultured on different occasions, reflecting an overall complex nature of colony formation.

S E C T I O N F O U R

RED BLOOD CELL REQUIREMENT
OF T LYMPHOCYTE COLONIES

4.1 INTRODUCTION

It was shown in section 2 that the growth of T lymphocyte colonies relied on the inclusion of sheep-RBC during the agar culture. As there was a direct relationship between the concentration of sheep-RBC and the number of colonies produced it would be justified to assume that these red cells were actively involved in the augmentation of T colony growth. This requirement for sheep-RBC has also been demonstrated by Claesson et al. (1977a) and Mercola and Cline (1979) while numerous reports have shown that sheep-RBC increase the blastogenic response of lymphocytes to PHA (Tarnvik, 1970; Johnson, Smith and Kirkpatrick, 1972; Kondracki and Milgrom, 1977). In addition Kondracki and Milgrom (1977) found that red cell stromata or sonicated red cells markedly increased PHA-responsiveness. This would suggest that the sheep-RBC membrane was the critical factor and the knowledge of T-lymphocyte:sheep-RBC interactions of E-rosette formation lends weight to this proposal. The experiments described herein were designed to ascertain the relationship between colony formation and the requirement for sheep-RBC with particular reference to the possible requirement for a specific contact recognition of sheep-RBC by human T lymphocytes.

4.2 METHODS

4.2.1 Preparation of Alsever's solution

Alsever's solution	glucose	2.05 g
	trisodium citrate	0.42 g
	sodium chloride	0.42
	H ₂ O	to 100 ml

Alsever's solution was always prepared fresh and sterilised by millipore filtration prior to use.

4.2.2 Red cell preparations

Sheep red blood cells in Alsever's solution were always obtained commercially. Mouse and rat red cells were freshly collected into PBS containing heparin following cardiac puncture while human red cells were obtained from the red cell rich layer of a peripheral blood Isopaque Ficoll gradient. Bovine red cells were collected by bleeding the animal (Daisy) into an equal volume of Alsever's solution. All the blood cells were washed three times with PBS prior to their use and incorporated into the agar medium at the final concentration of a 1% packed cell volume. The cultures were performed according to the routine method (Fig 2.A) with the single exception being that equivalent concentrations of different red cells were used.

4.2.3 Preparation of ghost red cells

4.2.3.1 Reagents

50P8	$\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$	83.0 mg
	Na_2HPO_4	1.3 g
	H_2O	to 100 ml

5P8 1/10 50P8

5P8-S 20 ml 50P8
20 ml 1.5M NaCl
 H_2O to 200ml

SDS-P	$\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$	59.6 mg
	Na_2HPO_4	93.1 mg
	SDS	1.5 g
	H_2O	to 100 ml

(SDS= sodium laurel phosphate)

4.2.3.2 Method

Ghosts were prepared from SRBC according to the method of Dodge et al. (1963). Two ml of packed SRBC were washed three times in 5P8-S and the buffy coat was removed after each wash. Ice cold 5P8 (30 ml) was added to the remaining button of red cells and the solution was constantly stirred for 30 min at 4°C. The lysate was added to pre-cooled centrifuge tubes and centrifuged at approximately 2500 x g for 20 min at 4°C. At the termination of the centrifugation the supernatants were removed, taking care not to lose the fluffy pellet of ghost cells from the bottom of the tubes. Once the supernatant was discarded the tubes were tilted to expose the small pellet of organelles which was removed while the fluffy ghost cell pellet was left behind. The remaining ghosts were resuspended in 5P8 and the entire centrifugation procedure was repeated to a total of three spins after which time they were resuspended in a small volume of normal saline. A 50 ul sample was diluted 1/20 with SDS-P and the protein content was calculated spectrophotometrically by measuring the OD at 280 nm. The concentration of protein was estimated from formula that 1 mg/ml protein = 0.66 OD at 280 nm which was previously determined from known concentrations of bovine serum albumin in the same buffer.

4.2.4 Preparation of red cell lysates

Thrice washed SRBC were finally diluted to a 50% packed red cell volume with PBS. The lysates were prepared by snap freezing the SRBC in liquid nitrogen (-190°C), immediately thawing at 56°C until the last remaining ice crystal was dissolved; this freeze/thaw procedure was repeated three times. Equivalent volumes of this preparation were used to replace the intact SRBC during the agar phase of the culture procedure.

4.2.5 Red cell absorption of agar

Agar was prepared and sterilised as a 1.2% solution in double distilled water. Equal volumes of double concentrated alpha-medium supplemented with 10% FCS were added to the liquid agar. The liquid agar medium was then mixed with packed SRBC at a final ratio of 1 volume SRBC to 3 volumes agar medium. This agar-medium-SRBC preparation was gently mixed for 1 h at 50°C . After the agar was absorbed the red cells were allowed 1 h to settle to the bottom of the tube at 50°C . The preparation was then centrifuged at approximately $500 \times g$ for 3 min; the supernatant containing the molten agar was removed and held at 40°C . PHA-treated cells and PHA (at final concentrations of 50 000/ml and 20 ug/ml respectively) were added to this absorbed agar and 1 ml volumes of this SRBC absorbed-agar-medium containing PHA and PHA-treated cells were incubated for the usual 7 day period. Intact SRBC were added to the above preparation at the final concentration of 1% to act as the positive control.

4.2.6 Double layered agar cultures

Agar bases contained equal volumes of double strength alpha-medium and 1.08% agar supplemented with SRBC at a final concentration of 1% packed cells. The agar was allowed to gel and overlayed with 1 ml agar medium containing 50 000 PHA-treated cells, 20 ug PHA and equal volumes of double strength alpha-medium (supplemented with 20% FCS) and 1.08% agar. The cultures were incubated for the usual 7 days.

4.3 RESULTS

4.3.1 Requirement for sheep-RBC

When red cells from various sources were used to supplement the agar medium colony growth was observed in all cases (Table 4.1). The colonies grown in the presence of sheep-RBC were generally larger than those grown in the presence of the other red cells.

4.3.2 Requirement for contact between red cells and lymphocytes

With the observation that the specific recognition of sheep-RBC by human T lymphocytes did not account for the red cell requirement, experiments were performed to determine if a non-specific contact phenomenon occurred.

4.3.2.1 Colony growth in the presence of ghost red cells

When ghost red cells (or red cell stromata) at protein concentrations ranging from 0.1 to 0.4 mg/ml replaced the intact sheep-RBC during the agar culture there was no colony growth. A few small clusters containing no more than 20 cells was occasionally observed.

TABLE 4.1

T lymphocyte colony promoting ability of red blood cells from different mammalian sources

Red cell source	Number of colonies
Sheep	567 \pm 42
Allogeneic	77 \pm 21
Autologous	110 \pm 26
Rabbit	62 \pm 18
Mouse	84 \pm 20
Bovine	337 \pm 15
PBS	0

Results represent the mean \pm standard deviation from triplicate cultures per 50 000 cells. A representative experiment is shown.

Table 4.2

Comparison of double and single agar layer techniques on T lymphocyte colony formation

EXP.NO	Number of colonies	
	Double layer	Single layer
I	420 \pm 72	511 \pm 110
II	328 \pm 80	234 \pm 67
III	34 \pm 15	567 \pm 42
IV	380 \pm 20	353 \pm 49

Results are the mean \pm standard deviation from triplicate cultures per 50 000 cells.

4.3.2.2 Colony growth from double layered agar cultures

As shown in Table 4.2 colonies developed when sheep-RBC were incorporated in an agar base and PHA-treated cells but no sheep-RBC were present in an agar overlayer. The colonies developed 3-dimensionally throughout this overlayer and therefore contact between red cells and proliferating cells was not necessary for their development. No colonies were seen in the agar underlayer.

4.3.3 Requirement for intact red cells

The previous results suggested that the red cells contain soluble factor(s) that might augment colony growth. When sheep-RBC lysates, obtained from freeze/thawing, replaced the intact red cells no colony growth was observed. Occasionally a few small clusters were seen to develop. When red cell lysates and ghost red cells were incorporated together no colony growth was observed.

4.3.4 Red cell absorption of agar

The possibility that the red cells absorbed toxic products that might have been present in the agar preparation was tested by preabsorbing the molten agar with intact red cells. When PHA-treated lymphocytes and PHA were incorporated into this red cell absorbed agar there was no colony growth. Colonies were seen to develop when this red cell absorbed agar was supplemented with intact sheep-RBC.

4.4 DISCUSSION

The enhancing abilities of sheep-RBC have been demonstrated in a number of in vitro assay systems. The growth of mouse plasmacytoma cells was first demonstrated when whole blood cells were incorporated in the agar medium (Metcalf, 1973). Mitogenic blast transformation has been shown to increase in the presence of RBC or

RBC stromata (Tarnvik, 1970; Johnson et al., 1972; Yachnin et al., 1972; Kondracki and Milgrom, 1977) while a number of T colony systems have documented an absolute requirement for the presence of sheep-RBC (Claesson et al., 1977a; Mercola and Cline, 1979). The role of the sheep-RBC in these systems is unclear. It has been demonstrated that T cells exposed to sheep-RBC have an increased ability to respond to TCGF (Larsson, Andersson and Coutinho, 1978). One may therefore speculate that the benefit of sheep-RBC may relate to the unexplained, but specific binding of human T lymphocytes to sheep-RBC which may trigger some form of cellular activation. This specific cell contact phenomenon was investigated by substituting the sheep-RBC with RBC from other sources and it was found that a variety of RBC were able to support colony growth (Table 4.1). Therefore the specific recognition of sheep-RBC by human T cells does not account for this activity.

The high concentration of red cells in the agar medium encourages a non-specific cell contact between red cells and lymphocytes. As all of the red cells tested supported colony growth it is possible that nonspecific cell contact could account for the colony supporting ability of the red cells. As an adjunct to this possibility it has been proposed that red cells bind and concentrate mitogen which is then presented to the proliferating lymphocytes (Lotze, Strausser and Rosenberg, 1980). The possibility that colony growth was due to mitogen presentation or cell contact was not borne out by the present work as colonies developed when the red cells and proliferating lymphocytes were physically separated through the use of a double agar culture system (Table 4.2). Further evidence that cell contact does not contribute to colony growth can be obtained from the experiments using ghost red cells as it was found that ghost cells (i.e. red cell

membranes) could not substitute for whole red blood cells (section 4.3.2.1). Lotze et al. (1980) also found that ghost cells could not substitute for intact sheep-RBC in a colony assay. In other words the red cells within the agar culture system do not assist colony growth by presenting mitogen to the proliferating cells nor do they function through cell contact phenomena.

From these findings it would appear that the red cells contain soluble factors or nutrients which diffuse through the agar and are utilised by the proliferating lymphocytes. As various red cells supported colony growth it can be concluded that the factor(s) responsible are present in mammalian red cells in general and not limited to a single species. Replacement of the intact red cells with red cell lysates did not support colony growth (section 4.3.3) nor was there any colony growth when both red cell lysates and red cell ghosts were included in the agar medium. This demonstrates that red cells must be intact to be of benefit to the colony forming cells.

The possibility that the RBC absorbed any toxic products that may have resided in the agar is refuted by the results obtained in section 4.3.4 as prior absorption of the agar with sheep-RBC did not allow colonies to grow. When fresh sheep-RBC were added to this absorbed agar colony growth returned to normal. This finding, however, does not preclude the red cells from absorbing toxic products that may arise during the culture period. In fact, according to the evidence presented by Aarden et al. (1982), it seems highly likely that this does occur. They found that when culture medium was exposed to light, oxygen radicals were produced which were toxic to T lymphocytes. This toxicity was removed by incubating the medium with erythrocytes or by culturing the T cells in the presence of erythrocytes. It is likely

that the red cells can protect the lymphocytes from the lethal effects of oxygen radicals via the red cells' system of glutathione enzymes and glucose-6-phosphate dehydrogenase activity which efficiently remove oxygen radicals both in vivo and in vitro. As these enzymes would be destroyed following freeze/thawing procedures it is not surprising that red cell lysates could not substitute for intact red cells.

The findings of this section that red cells are actively involved in colony growth (through a possible protection of lymphocytes from the toxic effects of oxygen radicals) and the proposed ability, obtained from liquid culture systems, that red cells present mitogen to proliferating lymphocytes suggests they may be more versatile in vivo than merely transporting oxygen throughout the blood stream. With these possibilities and the observation that red cells recognise the C3b and C4b complement components enabling adherence of red cells to antigen-antibody-complement complexes (Nussenzweig, 1975) the existence of an immune red-cell system, proposed by Siegel, Liu and Gleicher (1981) seems feasible.

4.5 SUMMARY

A number of in vitro culture systems incorporate sheep-RBC to promote more favourable conditions but the exact role of these red cells is poorly defined. The results described in this section were aimed at investigating the requirement for sheep-RBC in the T lymphocyte colony system. The results not only showed that sheep-RBC were able to augment colony production but that red cells from other sources were also effective and therefore the activity was not a unique property of sheep-RBC. Through double layer agar techniques it

was shown that the activity was not mediated through a membrane: membrane contact phenomenon nor did the red cells "present" mitogen to the reactive cells. Rather, they elaborated soluble factors which diffused through the agar. The inability of red cell lysates and/or red cell ghosts to support colony growth demonstrated that an effective elaboration required intact cells. The red cells did not absorb toxic products from the agar. It was possible that the red cells, through a system of enzymes, absorbed out toxic oxygen radicals which may have developed during the culture period.

S E C T I O N F I V E

T COLONY FORMATION AND THE
REQUIREMENT FOR ADHERENT CELLS

The involvement of macrophages and monocytes in a variety of in vitro and in vivo immune responses has been well established. In addition to carrying out phagocytosis, macrophages may initiate and regulate humoral and cellular immunity through antigen presentation and helper or suppressor activities. It is not surprising that these cells have also been implicated with T colony formation. Claesson et al. (1977b) found that colonies did not develop in the absence of adherent cells (monocytes). Other workers have reported similar findings (Gordon, King and Gordon-Smith, 1980; Pistoia et al., 1981; Rozenszajn et al., 1981). In contrast some authors have shown that adherent cells were not needed for colony production (Spitzer et al., 1980). Furthermore others have demonstrated that monocytes, or monocyte products, have an inhibitory effect on colony production (Bockman and Rothschild, 1979; Klein et al., 1982); the absolute requirement for monocytes is therefore disputed. The experiments described in this section were aimed at elucidating the requirement for adherent cells through the use of depletion experiments performed prior to either the liquid or agar cultures. With this method the need for adherent cells at early and/or late stages of colony growth could be clarified. It is important to point out that the method used for monocyte depletion relied on their ability to adhere to polystyrene culture dishes. While monocytes effectively attach to the dishes other cells, including T cells, might also attach and therefore it was necessary to monitor the recovered cell populations to protect against the possibility that a particular subpopulation of T cells was selectively removed. This was performed with the monoclonal antibodies

Leu 2a and Leu 3a which recognise the suppressor and helper phenotypes respectively. In addition the presence of monocytes was monitored by a monoclonal antibody which reacted with human monocytes.

5.2 METHODS

5.2.1 Adherent cell depletion

Adherent cells were separated from non-adherent cells by their ability to attach to polystyrene culture dishes. This was performed with freshly isolated peripheral blood mononuclear cells or mononuclear cells that had been exposed to PHA for 20 h. Throughout this section the term "adherent cell" refers to mononuclear cells that adhere to polystyrene culture dishes and, as monitored by an anti-monocyte antibody, incorporates all monocytes.

For unstimulated mononuclear cells the following method was used. Five million mononuclear cells were held in 5 ml alpha-medium supplemented with 5% FCS in 100 mm petri dishes for 45 min at 37°C in a fully humidified atmosphere containing 10% CO₂ in air. At the end of the culture the petri dish was rocked a number of times and the supernatant harvested. The petri dish was briefly washed with 1 ml cold 0.25% trypsin to remove any loosely attached non-adherent cells. The plate was then rinsed with 5-10 ml cold medium and the harvested cells were washed and concentrated by centrifuging and resuspending in fresh alpha medium.

For PHA-stimulated preparations the adherent cells were removed according to the abovementioned procedure but with the following modifications to overcome the leucoagglutinating properties of PHA. Extensive washing and shaking following the PHA preincubation culture was necessary to disrupt the PHA induced aggregates of

adherent and non-adherent cells. These PHA-treated cells were then incubated for 30 min at a final concentration of 2×10^6 /ml in 100 mm petri dishes. During this 30 min incubation the plate was shaken a number of times. The supernatant was harvested as above with the single exception that two washes with 0.25% trypsin were performed. The harvested non-adherent cells were then washed with cold medium, concentrated and counted. If an unsatisfactory cell yield was obtained the petri dish was again trypsinized and washed.

The non-adherent cells prepared before or after PHA stimulation were cultured according to the usual two step technique with duplicate rather than triplicate plates; this reduced the demand for mononuclear cells. Contaminating monocytes were sought according to section 5.2.2 and if >1% monocytes were seen in a non-adherent cell preparation then that particular experiment was terminated. In this way only highly purified preparations were utilised. The choice of 1% was an arbitrary one. It has been shown that 1% monocytes may still be effective (Maizel, 1979) and it would be reasonable to assume that such a purification would be possible given the present methodology. It is possible that concentrations lower than 1% may still function effectively.

5.2.2 Detection of monocytes and T lymphocyte subpopulations

Indirect fluorescent techniques using monoclonal antibodies and fluorescein coupled anti-rat globulins were used to identify blood monocytes or helper and suppressor (phenotype) T lymphocytes. The monoclonal antibody FMCl7, kindly provided by Dr. H. Zola, was used for identifying monocytes (Brooks et al., 1983) and the commercial antibodies Leu 2a and Leu 3a were used to identify suppressor and helper T lymphocytes respectively. Approximately 50 ul of cell

suspensions containing 50 000 mononuclear, or PHA-treated mononuclear cells were incubated in polystyrene tubes with 10 ul of the relevant monoclonal antibody (previously titrated for optimum labelling) for 30 min at 4°C. Following this reaction the cells were washed 3 times with cold PBS. After the third wash all remaining liquid was drained from the tube and 100 ul of FITC-conjugated rabbit anti-rat immunoglobulin (MF08; previously absorbed and centrifuged to remove aggregates) was added and incubated at 4°C for 30 min. The preparation was washed 3 times after this reaction. The remaining button of cells was resuspended in 50 ul PBS-glycerol (50% PBS : 50% glycerol) and wet mounted on clean glass microscope slides under a glass coverslip and examined by fluorescent microscopy. At least 200 cells were counted for Leu 2a and Leu 3a analysis whereas at least 400 cells were counted for FMCl7 analysis.

5.3 RESULTS

5.3.1 Surface marker analysis

When adherent cells were removed prior to the PHA pre-incubation culture there was no alteration in the Leu 2a/Leu 3a ratio (Table 5.1). From the 6 experiments shown in Table 5.1 it should be noted that on 2 occasions (experiments 5 & 6) the percentage FMCl7 positive cells for the non-adherent fraction exceeded 1%. Usually these experiments would have been terminated, however, as revealed later these cultures provided useful information.

Removal of adherent cells after the preincubation culture produced no significant alteration in the ratios of Leu 2a/Leu 3a positive cells for the mononuclear and the non-adherent cell preparations (Table 5.2). It is interesting to note that the

TABLE 5.1

Surface markers of mononuclear (MN) and non-adherent cells (NAC) when the adherent cells were removed prior to PHA-stimulation

EXP.NO	% Leu 2a		% Leu 3a		Ratio* Leu 2a/ Leu 3a		% FMCl7	
	MN	NAC	MN	NAC	MN	NAC	MN	NAC
1	26	20	47	36	0.55	0.56	10	0
2	14	25	59	58	0.24	0.43	13	1.0
3	n.d.		n.d.		n.d.		5	0
4	27	26	53	49	0.51	0.53	15	0.6
5	18	22	35	41	0.51	0.54	12	1.5
6	20	19	32	39	0.63	0.49	29	2.1

MN All mononuclear cells

NAC Mononuclear cells depleted of adherent cells

Leu 2a T lymphocytes of suppressor phenotype

Leu 3a T lymphocytes of helper phenotype

FMCl7 Blood monocytic cells

* Paired t-test revealed no significant difference ($p > 0.5$)

TABLE 5.2

Surface marker analysis of mononuclear (MN) and non-adherent cells (NAC) when the adherent cells were removed after PHA-stimulation

EXP.NO	% Leu 2a		% Leu 3a		Ratio* Leu 2a/ Leu 3a		% FMCl7	
	MN	NAC	MN	NAC	MN	NAC	MN	NAC
1	32	38	15	12	2.13	3.17	6	0
2	25	20	48	40	0.52	0.50	8	0
3	n.d.		n.d.		n.d.		1	0
4	42	36	28	25	1.50	1.44	7	0.5

MN All unseparated PHA-stimulated mononuclear cells

NAC Mononuclear cells depleted of adherent cells after PHA-stimulation

Leu 2a T lymphocytes of suppressor phenotype

Leu 3a T lymphocytes of helper phenotype

FMCl7 Blood monocytic cells

* Paired t-test revealed no significant difference ($p > 0.4$)

TABLE 5.3

T lymphocyte colony forming ability of unseparated mononuclear cells (MN) and mononuclear cells depleted of adherent cells (NAC) either prior to or following the PHA-stimulation of these cells in liquid culture

EXP.NO	Number of colonies							
	(liquid)		MN*		NAC'		MN''	
	(agar)		MN		NAC		NAC	
1			270	176	4	8	250	296
2			500	540	15	65	500	440
3			178	162	30	30	110	130
4			165	195	11	15	210	230

Results represent the number of colonies produced per 50 000 PHA-treated cells from duplicate plates.

* all mononuclear cells cultured throughout the liquid and agar cultures

' adherent cells removed prior to the liquid culture i.e. NAC incubated throughout the liquid and agar cultures

" adherent cells removed after the liquid culture i.e. mononuclear cells incubated during the liquid culture, adherent cells were then removed and these NAC were seeded into agar.

percentage of cells positive for Leu 3a and FMC17 decreased after the preincubation culture.

These results show that the method used for removing adherent cells (prior to and after the PHA preincubation culture) did not selectively eliminate a major subpopulation of T lymphocytes.

5.3.2 Colony growth after adherent cell depletion

Cell populations depleted of adherent cells prior to the 20 h preincubation culture did not develop into colonies. Adherent cell depletion performed after the 20 h preincubation culture had no affect on colony growth (Table 5.3) thus indicating that adherent cells were only essential at the early stages of colony development. It should be noted that the same experiment numbers (1-5) from Tables 5.1, 5.2 & 5.3 refer to the same experiment and therefore the same cell preparations.

From Table 5.3 two interesting points emerge. The first concerns experiment number 5. There was, in contrast to the general trend, good colony growth from the apparent non-adherent cell population. As shown in Table 5.1 these "non-adherent cells" prepared from experiment 5 still contained 1.5% FMC17 positive cells suggesting that the monocytes were effective at this concentration. The second point involved experiment number 6. For this example colony growth from the unseparated mononuclear cells was poor. Removal of most of the adherent cells resulted in good colony growth. Table 5.1 shows that this mononuclear cell preparation contained a high (29%) proportion of monocytes and the "non-adherent cell" population contained a lower, but positive, proportion (2.1%). This observation points to monocytes as being suppressive at high concentrations.

In previous sections of this thesis indirect evidence was presented suggesting that cooperation of helper or accessory cells was required for colony formation. Monocytes have been shown to be important for the stimulation of T cells by mitogens, antigens or allogeneic cells (Hersh and Harris, 1968; Rode and Gordon, 1974; Schmidtke and Hatfield, 1976; Lipsky, Ellner and Rosenthal, 1976; Nelson and Gatti, 1976; Rosenstreich, Farrar and Dougherty, 1976; Beller, Farr and Unanue, 1978; Passwell et al., 1982). Some investigators disagree and report that mitogen activation is either independent (Folch, Yoshinaga and Waksman, 1973; Waldrom, Horn and Rosenthal, 1974; Baird and Kaplan, 1977) or only partly dependent on monocytes (Lohrman, Novikous and Graw, 1974). There are several possibilities for these contradictions including variations in the source of monocytes and the separation procedures employed to obtain or deplete cell suspensions of monocytes. Alternatively, monocytes may be active at very low concentrations (Maizel et al., 1979) and appear to be required immediately after mitogen exposure (Mookerjee and Ballard, 1979). Monocytes can inhibit lymphocyte responses when either stimulated (Keller, 1975; Kurland and Bockman, 1978; Passwell, Dayer and Merler, 1979; Bockman and Rothschild, 1979) or in high concentrations (Passwell et al., 1982) and therefore exert both suppressive and stimulatory influences on T lymphocyte functions. A similar situation exists with T lymphocyte colony formation as some investigators have demonstrated an absolute dependence of colony production on either adherent cells (presumably monocytes) or their products (Claesson et al., 1977b; Gordon et al., 1980; Pistoia et al., 1981; Rozenszajn et al., 1981) while others have found that monocytes

are not needed and even suppress colony growth (Zeevi, Goldman and Rozenszajn, 1977; Bockman and Rothschild, 1979; Spitzer et al., 1980; Klein et al., 1982). In light of the abovementioned evidence it would be logical to predict that monocytes exert dual roles on colony growth.

The results summarised in Table 5.3 show that for the present work colony growth required the presence of adherent cells during the early stages of colony formation only; a finding that not previously described for T colony formation. It is recognised that these results, by themselves, do not exclude the possibility that the colony-forming cells were amongst the mononuclear preparation removed with the adherent cells. However experiments to be described in section 6 will show that, under appropriate conditions, the non-adherent cells could readily be induced to form colonies. Furthermore Claesson et al. (1977b) found that colonies developed from non-adherent cells provided a source of conditioned medium (prepared from adherent cells) was available. These findings suggest that the colony forming cells are contained within the non-adherent fraction of mononuclear cells. The absence of colony growth from the non-adherent cells (separated prior to PHA stimulation) was not due to a selective depletion of a subpopulation of T lymphocytes as the ratio of Leu 2a/Leu 3a cells was unaltered by the separation procedure (Table 5.1), nor was it due to the trauma of separation because the identical procedure, when performed with PHA-treated cells, had no affect on colony survival.

The monocytes within the adherent cell population were likely to be responsible for the colony promoting activity. Evidence for this derives from Tables 5.1 & 5.3 which show good colony growth

when the total fraction of monocytes (FMCl7) exceeded 1.5%, whereas there was only limited growth when the fraction of FMCl7 cells was below this value. Tables 5.1 & 5.3 also show that in the presence of 29% monocytes colony growth was extremely poor (experiment 6). When most of these monocytes were removed (apart from 2.1%) colony growth returned to normal; the implication being that a high concentration prevented colonies from forming but a low concentration was essential. With the evidence that monocytes suppress mitogen induced blast transformation at high monocyte:lymphocyte ratios (Passwell et al., 1982) it seems probable that a similar situation applies to T colony formation. Such a finding has been reported by Rozenszajn et al. (1981) who showed that low numbers of monocyte-macrophages were essential for colony growth whereas excessive numbers were inhibitory.

It will be recalled from section 2 that colonies were produced when E-rosette enriched cells were cultured. It might be supposed that these cells were a pure T cell population without adherent cells or monocytes. Nevertheless the single separation of E-RFC was unable to produce a pure population and a small proportion of contaminating cells, including monocytes, was present. It appears likely that a number of the published results stating that monocytes are not required for colony growth overlooked the possibility that minor concentrations of monocytes were sufficient for a proliferative response.

A number of authors have reported contradictory findings concerning the requirement of colony production on a source of adherent cells (or monocytes). The role of adherent cells was investigated in this section by removing them prior to or following the liquid culture stage. Through these depletion studies and by monitoring the remaining population with an anti-monocyte antibody it was shown that adherent cells (presumably the monocyte fraction) were essential for T lymphocyte colony formation. These cells were active at very low concentrations and their role was evident during the early stages of colony development only. It was also found that monocytes could exert inhibitory influences when in high concentrations and therefore exerted dual roles according to their concentration.

SECTION SIX

THE ROLE OF INTERLEUKIN 2 IN T LYMPHOCYTE COLONY FORMATION

It has been established with murine liquid culture systems that T cell activation requires both antigen (mitogen) and costimulator or Interleukin 1 (IL1) activity (Talmage et al., 1977; Shaw et al., 1978); proliferation of these activated cells is dependent on a maintenance or Interleukin 2 (IL2) activity (Lafferty, Andrus and Prowse, 1980). Thus T cell responses can be described by two distinct reactions namely 1) activation ($T \rightarrow T'$) and 2) proliferation ($T' \rightarrow nT'$). To test the hypothesis that the agar stage of colony development corresponded to the second reaction the role of a lymphokine with IL2 activity was investigated. In addition this section details experiments designed to determine whether the reactions described for in vitro liquid cultures could be demonstrated in the T lymphocyte colony culture system. From the findings a model describing the cellular interactions of colony formation is proposed.

The microfilament-microtubule protein system of the cell constitutes a cytoskeletal control mechanism governing the movement and formation of receptor protein complexes which in turn provide signals for the nucleus to control cellular activity (Nicolson, 1976; Schreiner and Unanue, 1976). Since receptors for PHA and IL2 have been recognised it is likely that both PHA and IL2 molecules function through their respective receptors and the microfilament-microtubule system. This hypothesis was tested with the microfilament disrupting agent cytochalasin B (CB) by adding it at either the activation or proliferation phase in the presence of PHA or IL2 respectively.

6.2 METHODS

6.2.1 Source of the IL2 preparation

The IL2 used in this section was kindly provided as a lymphokine preparation with IL2 activity by Dr. H.S. Warren of the Cancer Research Unit, Woden Valley Hospital, Woden, A.C.T. Its method of preparation has been fully described elsewhere (Warren and Pembrey, 1981). Briefly, mononuclear cells from human tonsils were collected aseptically into sterile medium, washed in fresh medium after cell clumps had settled to the bottom of the tube and the supernatant containing the suspended cells harvested. The lymphocyte preparation (at $3-6 \times 10^8$ cells in 15 ml) was incubated for 2 h with PHA-containing, serum-free medium in plastic tissue culture flasks of 75 cm² surface area. Unbound PHA was removed by washing the cell suspensions and the cultures were incubated for a further 17 h in 30 ml of serum-free medium. The supernatant was collected by centrifugation, concentrated 10-fold over an Amicon YM-10 membrane and centrifuged to remove any insoluble material. The concentrated supernatant was further purified on hydroxylapatite and Sephadex G-25 columns. The IL2 activity was demonstrated using maintenance assays (Warren and Pembrey, 1981). This lymphokine will, for brevity be referred to as "IL2" throughout this work, with the reservation that it is not necessarily a pure preparation of IL2-proteins.

6.2.2 Incorporation of IL2 into the culture system

To investigate the stage(s) of colony development that IL2 acts the IL2 preparation was incorporated instead of PHA during either or both the liquid preincubation and agar cultures. Unless otherwise stated the concentration of the IL2 preparation was 1/100, previously

shown to be optimal for maintenance assays (Warren and Pembrey, 1981) while the concentration of PHA was 20 ug/ml. All colony assays (6.2.2 - 6.2.6) were performed according to the final technique outlined in section 2.3 (scheme a of Table 6.A) with the following modifications.

6.2.2.1 Incorporation of IL2 in the agar ($T' \rightarrow nT'$) culture.

IL2 was incorporated into the agar culture phase by substituting the IL2 preparation for PHA (scheme b of Table 6.A). Control cultures with medium replacing PHA and IL2 during the agar phase were always performed in parallel (scheme c of Table 6.A).

6.2.2.2 Incorporation of IL2 during the preincubation ($T \rightarrow T'$) culture.

IL2 was incorporated in the liquid preincubation culture by substituting PHA, during this step only, with the IL2 preparation (scheme d of Table 6.A). Control cultures with medium alone replacing PHA and IL2 during the liquid phase were always performed in parallel (scheme e of Table 6.A).

6.2.2.3 Incorporation of IL2 throughout both the liquid and agar cultures ($T \rightarrow T' \rightarrow nT'$)

IL2 was substituted for PHA during both the liquid and agar cultures (scheme f of Table 6.A). Positive control cultures (scheme b of Table 6.A) were always performed in parallel.

6.2.2.4 Incorporation of IL2 and PHA in the preincubation ($T \rightarrow T'$) culture

Mononuclear cells were cultured in the presence of both PHA and IL2 during the preincubation phase prior to seeding into IL2- or PHA-containing agar (schemes g and h of Table 6.A respectively).

Table 6.A

Schemes for incorporating PHA and IL2 into the colony assay

scheme	liquid phase	agar phase
	20 h (T \rightarrow T')	7 d (T' \rightarrow nT')
a	PHA	PHA
b	PHA	IL2
c	PHA	MEM
d	IL2	PHA
e	MEM	PHA
f	IL2	IL2
g	PHA + IL2	PHA
h	PHA + IL2	IL2
i	PHA	PHA + IL2

PHA: 20 ug PHA per ml unless otherwise stated

IL2: 1/100 final concentration unless otherwise stated

MEM: medium alone

6.2.2.5 Incorporation of both PHA and IL2 during the agar culture ($T' \rightarrow nT'$)

IL2 and PHA were incorporated together during the agar culture phase according to scheme i of Table 6.A.

6.2.3 Cell dose response curves produced in the presence of IL2 or PHA.

PHA treated cells, at varying concentrations, were seeded into PHA- or IL2-containing agar. The number of colonies was plotted against the corresponding number of cells plated. A computer defined line of best fit was calculated by considering all the points rather than their mean values. This line was extrapolated to zero cells plated to determine the X-axis intercept and the correlation coefficient was determined by a computer with a linear regression program. The cell dose response curve was also plotted on log axes. For this line the correlation and regression coefficients as well as the line of best fit were determined as above by transforming the data into their log values and entering this data into a computer with a linear regression program.

6.2.4 PHA and IL2 cell pulsing experiments

Mononuclear cells were incubated with PHA for periods ranging from 5 min to 20 h. Following this incubation the treated cells were washed free of unbound PHA and seeded into either PHA- or IL2-containing agar. Direct plating was performed by seeding untreated mononuclear cells directly into agar containing both PHA and IL2. Colony growth was scored after the cells had been cultured in agar for 7 days.

Pulsing of 20 h PHA-treated cells prior to seeding into agar medium was performed by incubating these treated cells for 1 h with either PHA or IL2. At the termination of this "pulse" the cells

were seeded into agar medium from which both PHA and IL2 were omitted.

6.2.5 Adherent cell depletion experiments

Cells that adhered to plastic culture dishes were removed according to the method described in section 5.2.1. Contaminating monocytes were sought by fluorescence microscopy and a monoclonal anti-monocyte antibody as described in section 5.2.2. (As with section 5.2.2 the demonstration of contaminating monocytes resulted in the termination of that particular experiment). The cell suspensions, depleted of adherent cells, were incubated with PHA throughout the usual 20 h preincubation culture, after which time they were seeded into either PHA- or IL2-containing agar. Positive control cultures (untreated mononuclear cells) were always prepared in parallel.

6.2.6 Treatment of cells with cytochalasin B

6.2.6.1 Preparation of cytochalasin B solution

Cytochalasin B (CB) was dissolved in dimethyl sulphoxide (DMSO) and stored as a stock solution of 5 mg/ml. Prior to use the CB solution was diluted to a final concentration of 0.5 mg/ml in alpha medium.

6.2.6.2 Cytochalasin B treatment

The effect of CB on the activation of resting T cells ($T \rightarrow T'$) was determined by incubating the drug at the final concentration of 10 ug/ml (previously shown not to affect cell viability (Woods and Boyd, 1982)) with 1×10^6 mononuclear cells, in the absence of PHA, for an initial period of 1 h. PHA was then added to give a final concentration of 20 ug/ml and these drug-treated cultures were incubated for a further 20 h prior to seeding in PHA- or

IL2-containing agar according to the standardised technique. DMSO control cultures were always performed in parallel by substituting the corresponding concentration of DMSO for the CB. To provide a control for the possibility of colony development due to limited activation during the agar culture after CB withdrawal, parallel cultures incubated with medium alone during the preincubation period were set up and the resulting cells seeded into either PHA- or IL2-containing agar.

The effect of CB on the conversion of activated T cells into colonies ($T' \rightarrow nT'$) was determined by seeding PHA-treated cells into either PHA- or IL2-containing agar in the presence of 10 ug/ml CB. DMSO control cultures were always performed in parallel.

6.2.7 PHA-induced blast transformation

Mononuclear cells at a final concentration of 1×10^6 /ml were suspended in alpha medium supplemented with 5% FCS. Volumes of 0.1 ml of this suspension were added to the wells of round bottomed microtitre plates and PHA or IL2 was added at the final concentrations of 50 ug/ml and 1/100 respectively. The volume of each well was adjusted to 0.2 ml with alpha medium supplemented with 5% FCS and the microtitre plates were incubated for 44 h at 37°C in a fully humidified atmosphere of 10% CO₂ in air. Tritiated thymidine (10 ul containing 0.5 uCi: TRA 310 Amersham) was then added and the incubation continued for a further 4 h. At the end of this culture period the cell suspensions were harvested onto glass fibre paper discs with a cell culture harvester (Titertek: Flow) and washed extensively with distilled water. After the samples were dried (room temperature for 24 h or 56°C for 1 h) they were placed in scintillation vials containing 1 ml of scintillation fluid (liquifluor). Radioactivity was

measured in a liquid scintillation counter and the results were expressed as counts per minute (cpm) for each vial.

6.2.8 Statistical analysis

Paired t-tests were always performed on the mean number of colonies produced from replicate plates. A computer with a paired t-test program was used to perform the analysis. It should be noted that the same experiment numbers (1-16) from Tables 6.1, 6.2 and 6.3 refer to the same cell preparations performed in parallel.

6.3 RESULTS

6.3.1 Stages of IL2 activity in colony formation

6.3.1.1 IL2 incorporated during the agar (proliferative; $T' \rightarrow nT'$) culture

When IL2 replaced PHA during the proliferative or agar phase of colony growth there was an overall increase in both the number of colonies per plate and the average size of these colonies. Table 6.1 shows colony numbers from 15 experiments when either PHA, IL2 or medium alone was incubated with PHA-activated cells during the agar phase. It can be seen that IL2 supported the proliferation into colonies of PHA-activated cells and, as assessed by an arbitrary visual scale (+ ++ +++), the colonies were consistently larger than their counterparts cultured in the presence of PHA. Furthermore, when colony growth was poor in the presence of PHA (expts. 2, 4 & 15) the corresponding cultures with IL2 yielded normal numbers of colonies. Fig 6.1 demonstrates graphically the increased number of colonies produced in the presence of IL2; a paired t-test revealed a significant difference between colony numbers in PHA compared to IL2 cultures ($p < 0.01$). It should be noted that the paired t-test was also

Table 6.1

Colony growth when PHA-treated cells were seeded into either PHA-, IL2-, or MEM- containing agar

EXP.NO	Number of colonies*		
	(liquid) PHA	PHA	PHA
	(agar) PHA	IL2	MEM
1	180 300 (+)	420 431 (+++)	7 12
2	10 30 (++)	300 300 (+++)	6 7
3	500 390 (+)	520 430 (+++)	100 130
4	30 30 (+)	760 640 (+++)	2 2
5	500 460 (+)	700 610 (++)	100 150
6	220 250 (+)	480 420 (+++)	0 0
7	450 540 (++)	390 410 (+++)	7 0
8	325 220 (++)	600 520 (++)	65 17
9	138 106 (+)	380 400 (++)	0 0
10	108 170 (+)	240 160 (+)	0 0
11	300 200 (+)	400 360 (+)	0 0
12	190 170 (+)	350 388 (++)	0 0
13	300 380 (+)	296 360 (+)	0 0
14	300 240 (+)	440 480 (++)	12 10
15	45 13 (+)	225 210 (+)	0 2

* Number of colonies per 50 000 PHA-treated cells from duplicate plates for each experiment.

(+) - approx. 50-100 cells per colony

(++) - approx. 100-200 cells per colony

(+++)- more than 200 cells per colony

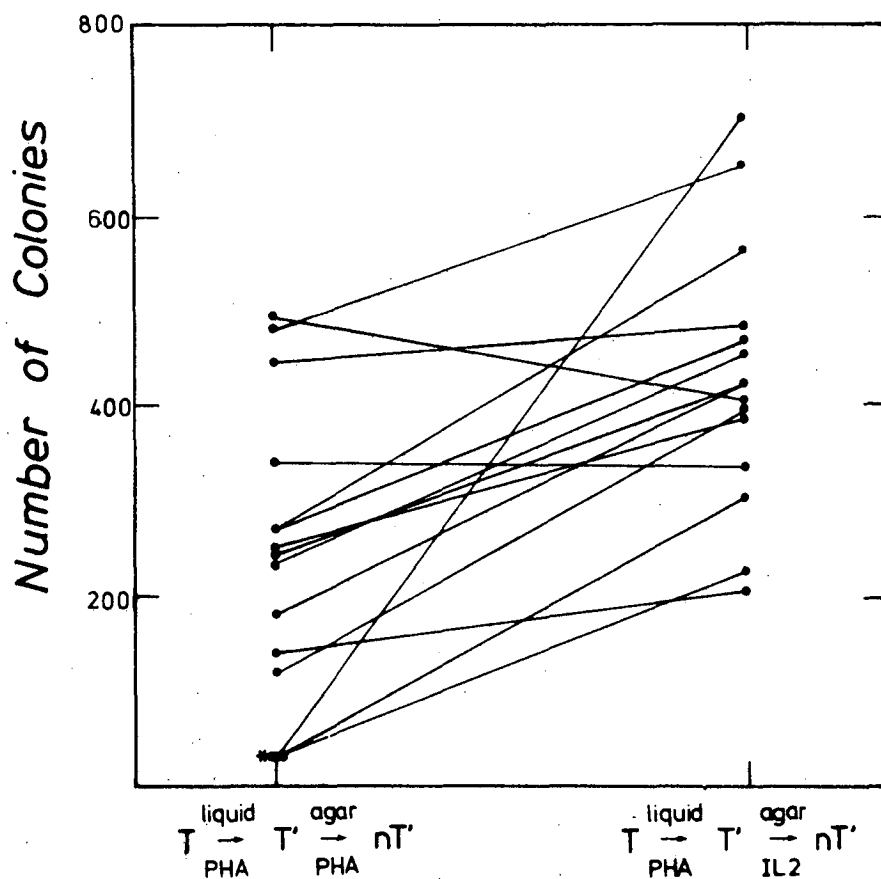


Fig 6.1

Comparison of the number of colonies produced when 50 000 PHA-treated cells were seeded into agar containing PHA or IL2. Each point represents the mean of duplicate plates. A significant difference in the number of colonies was revealed when all pairs were included in a paired t-test ($p < 0.01$). When the pairs corresponding to the PHA-nonresponders (*) were omitted from the paired t-test there was still a highly significant difference ($p < 0.01$) in the number of colonies.

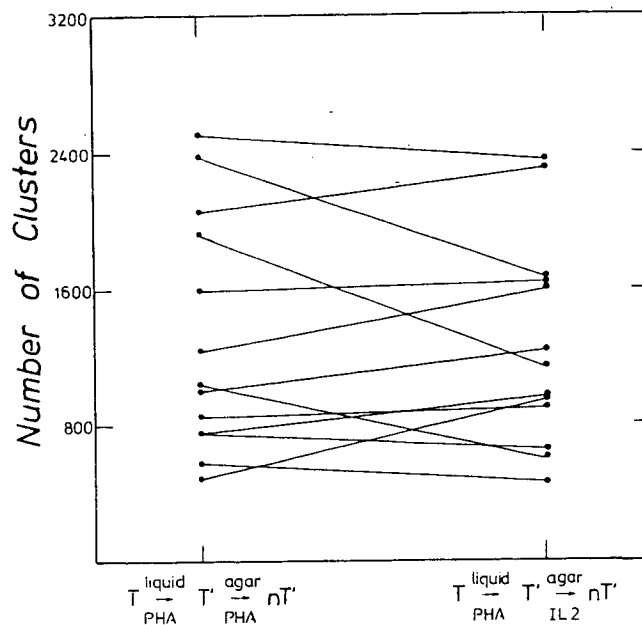


Fig 6.2

Comparison of the number of clusters produced when 50 000 PHA-treated cells were seeded into agar containing PHA or IL2. Each point represents the mean of duplicate plates. There was no significant difference in the number of clusters from either system as revealed by the paired t-test ($p > 0.5$).

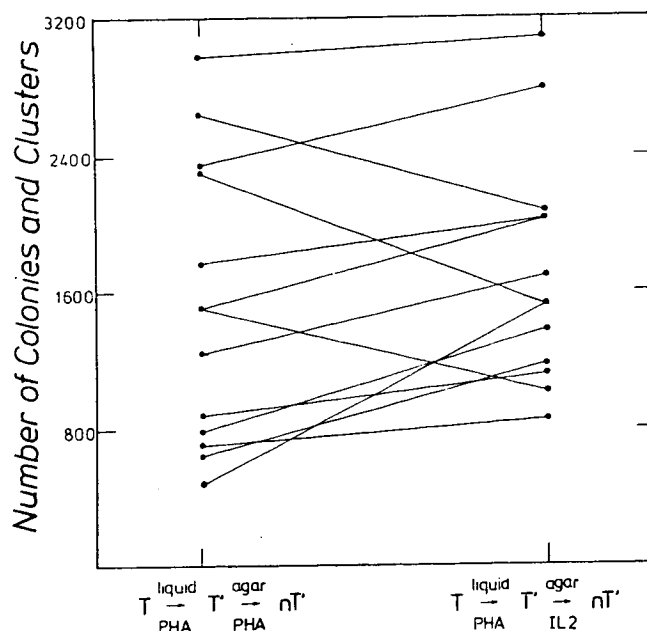


Fig 6.3

Comparison of the total number of colonies plus clusters when 50 000 PHA-treated cells were seeded into agar containing PHA or IL2. Each point represents the mean of duplicate plates. There was no significant difference in the number of colonies plus clusters in either system as revealed by the paired t-test ($p > 0.1$).

performed on all pairs excluding the 3 that corresponded to the PHA non-responders (* of Fig 6.1) and again there was a significant difference in the number of colonies ($p < 0.01$) demonstrating that the occasional absence of colonies in the PHA system did not unrealistically weigh the t-test in favour of the IL2 system. Although IL2 increased the total number of colonies there was no corresponding increase in the number of clusters (Fig 6.2; $p > 0.5$) nor was there an increase in the total number of colonies plus clusters combined (Fig 6.3; $p > 0.5$). An observation from Table 6.1 is that, on occasions, colonies could develop without either PHA or IL2 during the agar culture.

6.3.1.2 IL2 incorporated during the liquid (activation; $T \rightarrow T'$) culture

Mononuclear cells incubated with IL2 during the preincubation culture occasionally divided to form colonies when seeded into PHA-containing agar (Table 6.2). This was observed in 4/10 cultures while the medium controls (mononuclear cells preincubated with medium alone prior to seeding into PHA-containing agar) produced no colonies and the corresponding PHA controls produced colonies in all 10 experiments.

The activity of IL2 during the preincubation phase was further investigated by seeding IL2-treated cells into agar containing varying concentrations of PHA. The results summarised in Fig 6.4 demonstrate that cells treated with IL2, and unable to proliferate in the presence of PHA, cannot be induced to form colonies with increased concentrations of PHA.

Table 6.2

Colony growth when either PHA-, IL2- or MEM- treated mononuclear cells were seeded into PHA-containing agar

EXP.NO	Number of colonies*					
	(liquid)		IL2		MEM	
	(agar)	PHA	PHA	PHA	PHA	PHA
3		500 390	10 41		20 30	
5		500 460	0 10		2 0	
6		220 250	380 400		0 0	
7		450 540	200 150		49 36	
9		138 106	230 200		0 0	
10		300 200	0 0		0 5	
12		190 170	60 80		50 70	
13		300 380	400 320		3 6	
14		300 240	12 0		0 0	
17		750 610	0 7		7 3	

*Number of colonies produced per 50 000 PHA-treated cells from the duplicate plates for each experiment.

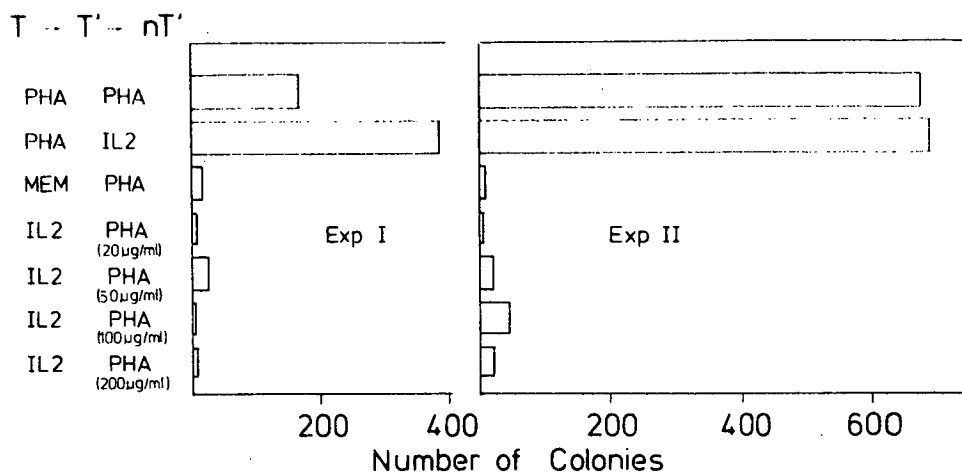


Fig 6.4

Colony formation of IL2-treated cells when seeded into agar containing varying concentrations of PHA. Results represent the mean number of colonies from duplicate plates per 50 000 cells seeded for each experiment. Unless shown PHA was used at a final concentration of 20ug/ml and IL2 was used at 1/100.

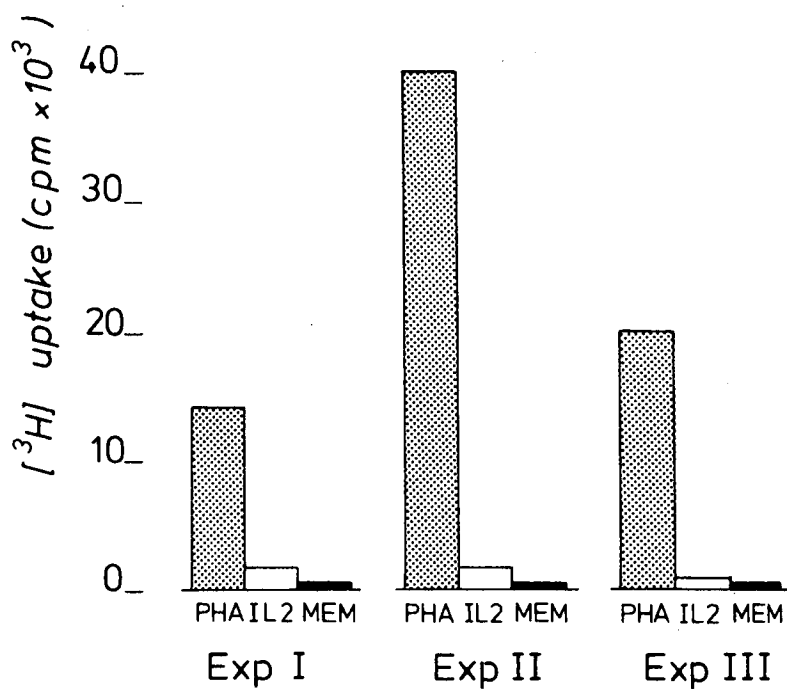


Fig 6.5

Mitogenic activities of PHA, IL2 and MEM. Columns represent the mean of duplicate cultures from each experiment.

6.3.1.3 Incorporation of IL2 throughout both the preincubation and agar cultures ($T \rightarrow T' \rightarrow nT'$)

On finding that the IL2 preparation was sometimes active during the liquid phase further experiments were performed whereby IL2 treated cells were seeded into IL2-containing agar and assayed for colony formation. As shown in Table 6.3 IL2 alone was unable to activate and support the conversion of resting cells into colonies. This is more clearly seen by following the results of experiment 13 documented in Tables 6.1, 6.2 and 6.3, since both protocols (IL2 preincubation/IL2 proliferation versus IL2 preincubation/PHA proliferation) were performed in parallel. This experiment showed that the activity of the IL2 preparation was not demonstrated unless PHA was present during the agar culture.

6.3.2 IL2-induced and PHA-induced blast transformation

The results summarised in Fig 6.5 show that the IL2 preparation was unable to initiate and support cellular proliferation in a liquid culture system as there was negligible blast transformation when mononuclear cells were cultured with IL2.

6.3.3 Colony assay: IL2 and PHA dose response experiments

The ability of PHA or IL2 to support the proliferation of PHA-stimulated lymphocytes was found to be concentration dependent (Fig 6.6). It can be seen from Fig 6.6A that the maximum activity of IL2 occurred at the final concentration of 1/100. The peak response for PHA was within the range of 20 - 50 ug/ml and doses of 250 ug/ml were usually preventive (Fig 6.6B; section 2.3.1). The superiority of IL2 in maintaining colony growth was further recognised as the maximum number of colonies with IL2 usually exceeded the maximum number in the

Table 6.3

Colony growth when cells treated with either PHA or IL2 were seeded into IL2 containing agar

EXP.NO	(liquid) (agar)	Number of colonies*			
		PHA		IL2	
		IL2		IL2	
3		520	430	12	15
4		760	640	35	8
5		700	610	0	0
10		240	160	0	0
11		400	360	3	2
12		350	388	22	54
13		296	360	23	4
14		440	480	16	3
16		205	280	0	0

*Number of colonies produced per 50 000 PHA- or IL2-treated cells seeded in IL2-containing agar. Results from duplicate plates.

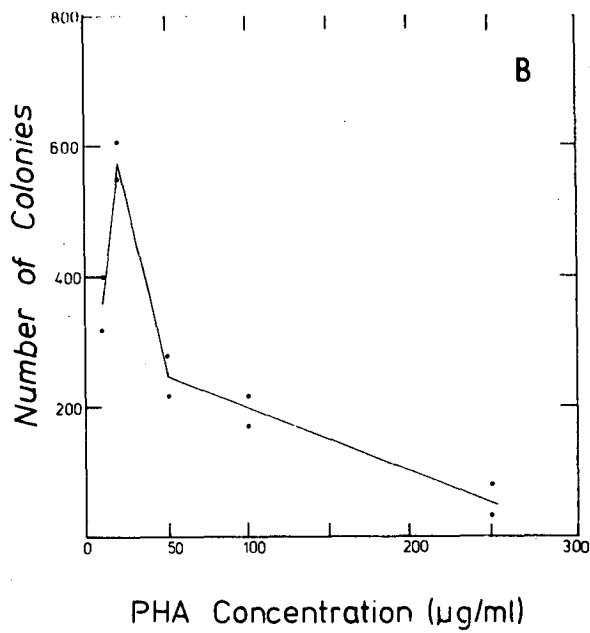
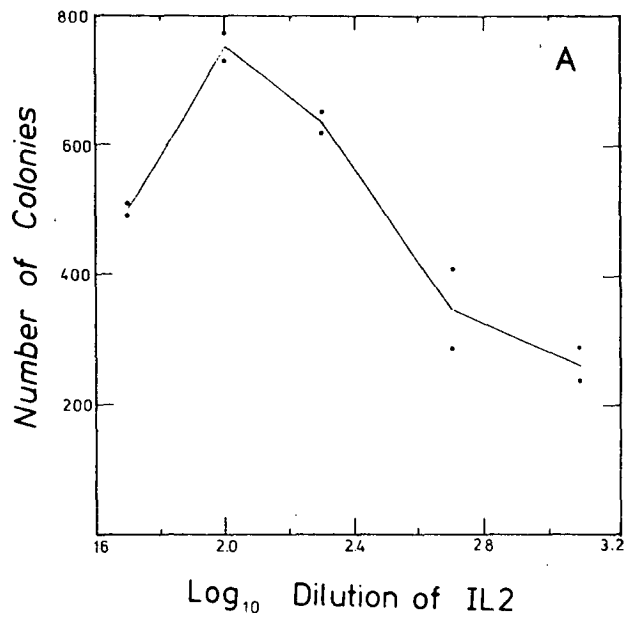


Fig 6.6(A and B)

Dose response titration curves for IL2 (top) and PHA (bottom). Each point represents the number of colonies produced per 50 000 PHA-treated cells seeded for each of the duplicate cultures from a representative experiment.

presence of PHA. In this series of experiments cells from one subject formed low numbers of colonies at all concentrations of PHA whereas colony growth in the presence of IL2 was normal (Fig 6.7); i.e. the lack of colony growth was not due to an inappropriate PHA concentration. Such subjects may be considered as "PHA non-responders".

6.3.4 Additive effects of PHA and IL2 on colony growth

Since either IL2 or PHA was shown to be active during both the activation and proliferative phases of T colony growth, experiments were conducted to ascertain whether their effects were additive or synergistic.

The results illustrated in Fig 6.8 do not show any additive effect when PHA-treated cells were seeded into agar containing both PHA and IL2; the number of colonies produced in the presence of IL2 alone was equivalent to the number of colonies produced when both IL2 and PHA were incorporated in the agar culture. This was also the case if the number of clusters (Fig 6.9) or the total of clusters plus colonies (Fig 6.10) was considered. An additive effect was, however, demonstrated when both IL2 and PHA were present during the preincubation culture. This increase in colony numbers was evident when the cells were plated into agar medium containing either PHA (Fig 6.11A; $p < 0.05$) or IL2 (Fig 6.11B; $p < 0.01$). In addition the colonies were generally larger after being preincubated with both PHA and IL2. There was no additive effect when the number of clusters (Fig 6.12) or the total of colonies plus clusters (Fig 6.13) was considered.

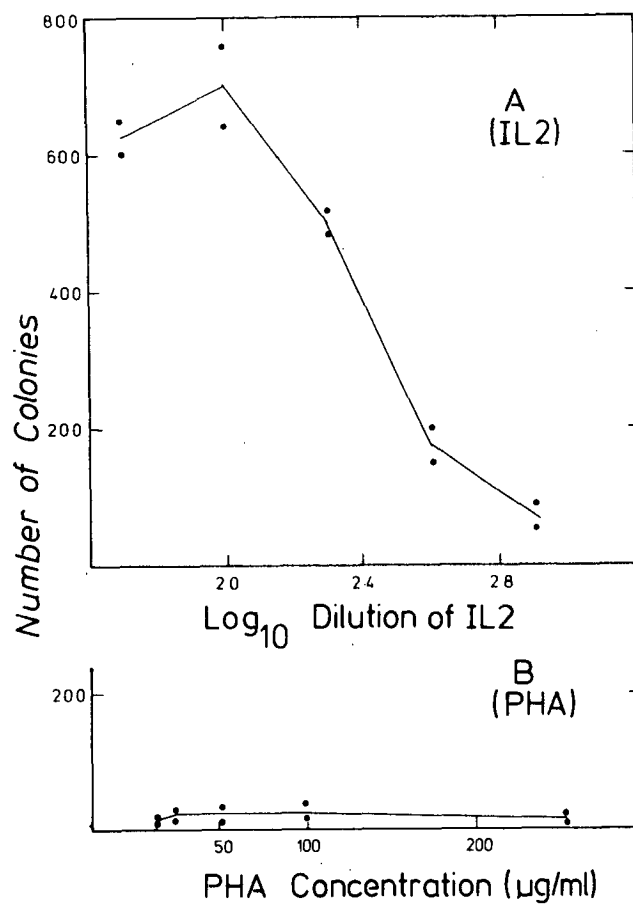


Fig 6.7(A and B)

Dose response titration curves for IL2 (top) and PHA (bottom) from a "PHA-nonresponder." Each point represents the number of colonies produced per 50 000 PHA-treated cells seeded for each of the duplicate cultures.

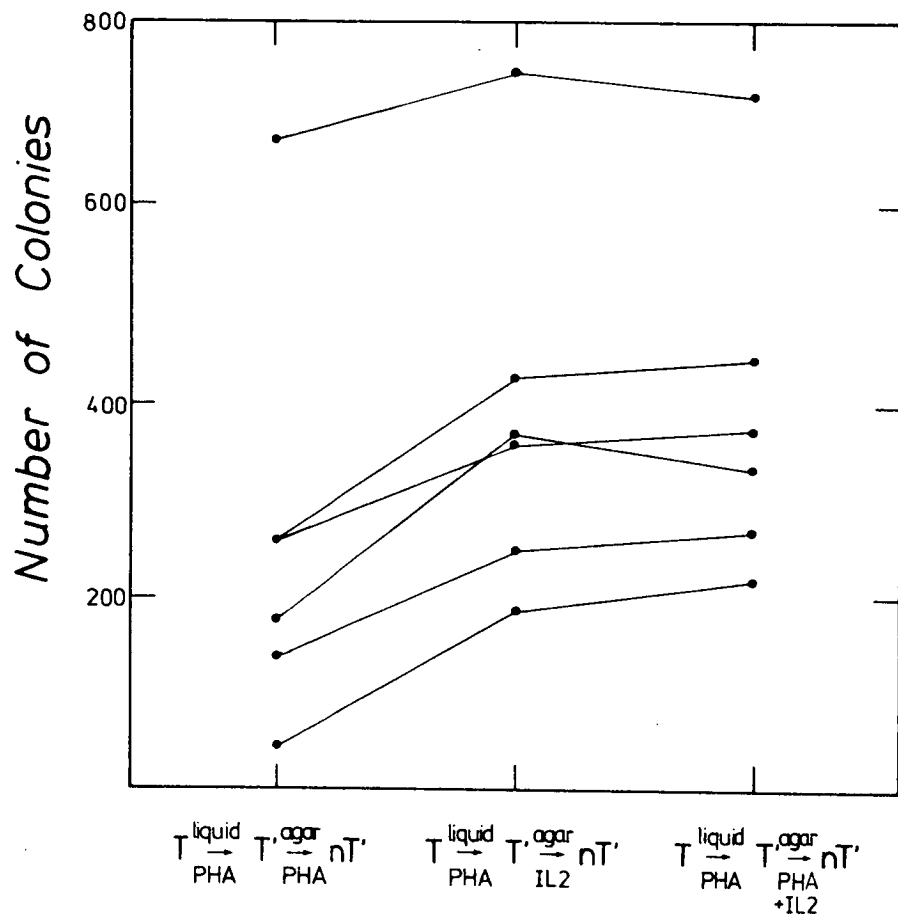


Fig 6.8

Comparison of the number of colonies produced when 50 000 PHA-treated cells were seeded into agar containing either PHA, IL2 or both PHA and IL2. Each point represents the mean number of colonies from duplicate plates. There was no significant difference when the number of colonies produced in the presence of IL2 alone were compared to those produced with both PHA and IL2 together ($p > 0.5$). As anticipated from Fig 6.1 there was a significant difference between the number colonies produced in the presence of PHA compared to IL2 alone ($p < 0.01$) and with PHA compared to both PHA and IL2 combined ($p < 0.01$).

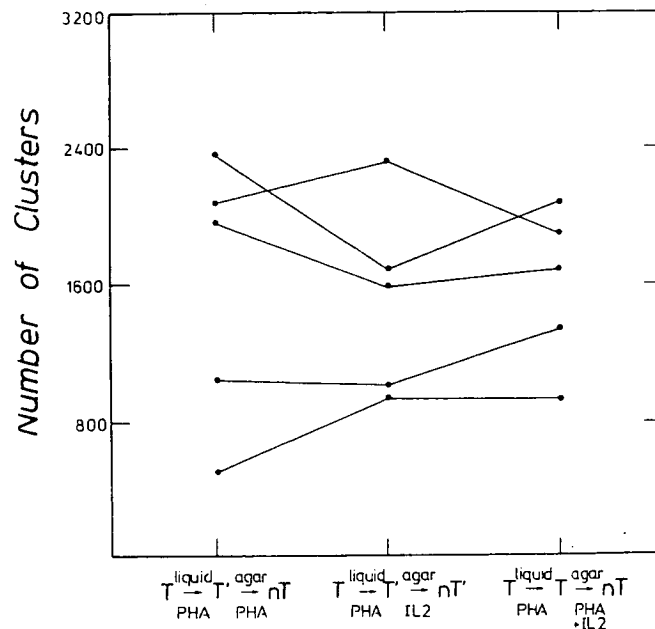


Fig 6.9

Comparison of the number of clusters produced when 50 000 PHA-treated cells were seeded into agar containing either PHA, IL2 or both PHA and IL2. Each point represents the mean number of clusters from duplicate plates for each experiment. No significant difference was revealed when all 3 groups were analysed by the paired t-test ($p>0.5$).

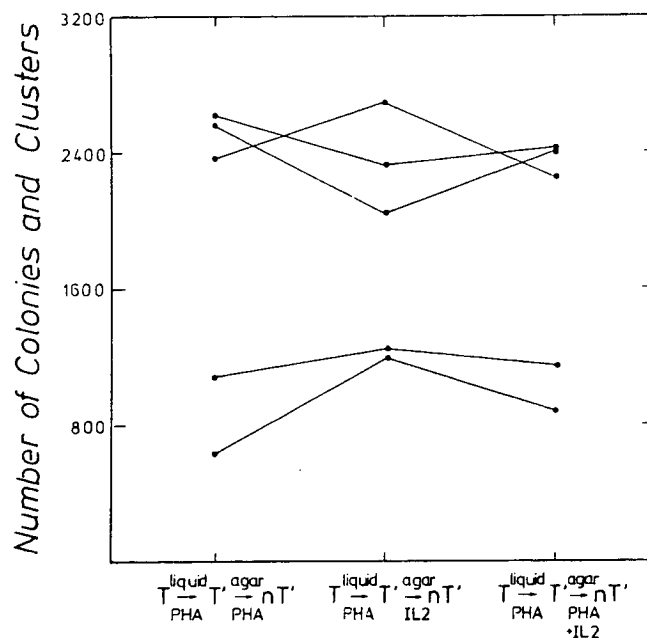


Fig 6.10

Comparison of the total number of colonies plus clusters produced when 50 000 PHA-treated cells were seeded into agar containing either PHA, IL2 or both PHA and IL2. Each point represents the mean number of colonies plus clusters from duplicate plates for each experiment. No significant difference was revealed when all 3 groups were analysed by the paired t-test ($p>0.5$).

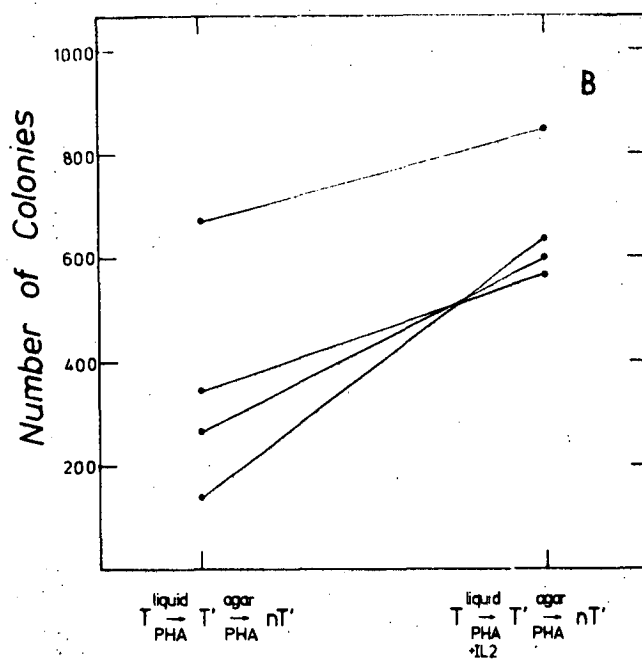
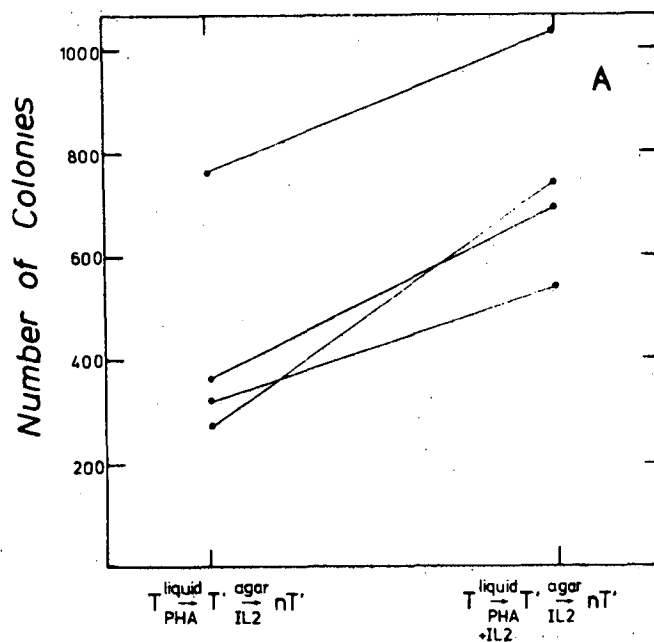


Fig 6.11(A and B)

Comparison of the number of colonies produced when 50 000 cells, treated with both PHA and IL2 together, were seeded into agar containing either IL2 (top) or PHA (bottom). Each point represents the mean number of colonies from duplicate plates for each experiment. When analysed by the paired t-test a significant difference in the number of colonies was revealed for both series of experiments ($p < 0.01$, Fig 6.11A; $p < 0.05$, Fig 6.11B)

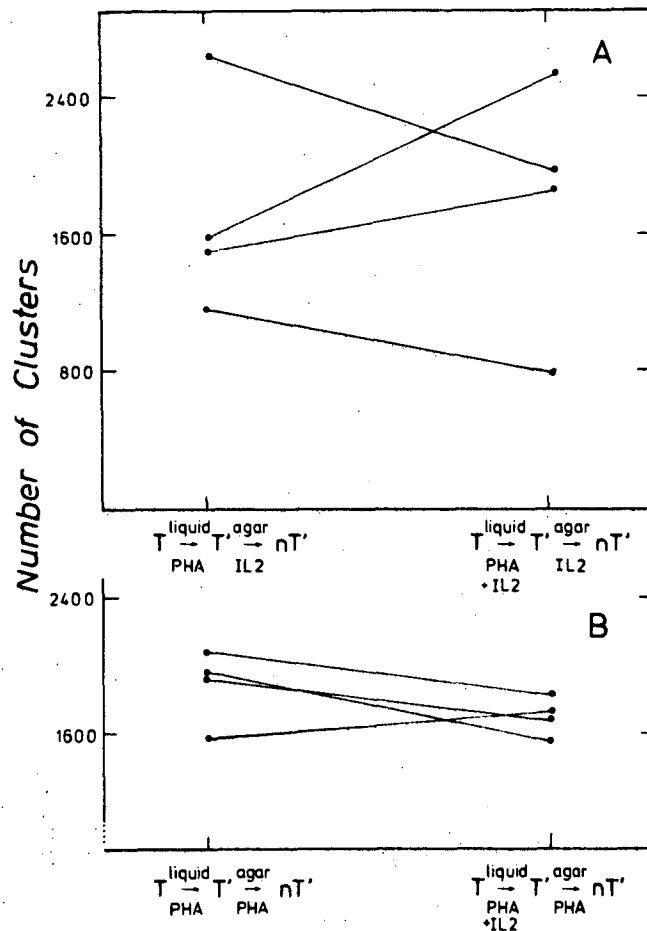


Fig 6.12(A and B)

Comparison of the number of clusters produced when 50 000 cells, treated with both PHA and IL2 together, were seeded into agar containing either IL2 (Fig 6.12A) or PHA (Fig 6.12B). Each point represents the mean number of clusters from duplicate plates for each experiment. When analysed by the paired t-test no significant difference in the number of clusters was revealed for both series of experiments ($p>0.5$, Fig 6.12A; $p>0.1$, Fig 6.12B)

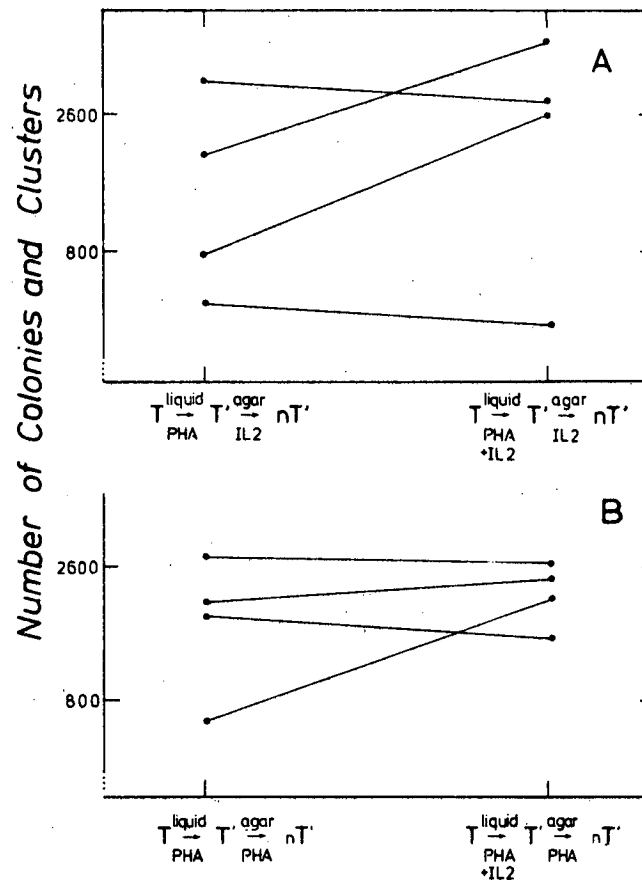


Fig 6.13(A and B)

Comparison of the total number of colonies plus clusters produced when 50 000 cells, treated with both PHA and IL2 together, were seeded into agar containing either IL2 (Fig 6.13A) or PHA (Fig 6.13B). Each point represents the mean number of colonies plus clusters from duplicate plates for each experiment. When analysed by the paired t-test there was no significant difference for both series of experiments ($p > 0.1$, Fig 6.13A; $p > 0.5$, Fig 6.13B)

6.3.5 Analysis of cells versus colonies: Cell dose response curves in the presence of IL2 or PHA

To determine the relationship between colonies and the number of cells plated, colony counts were plotted against the number of cells plated for PHA-treated cells seeded into either PHA- or IL2-containing agar. As Fig 6.14A demonstrates, colony growth in the presence of IL2 was directly proportional to the number of cells seeded and the straight line, when extrapolated to "zero colonies" intersected the X-axis near the origin. When PHA-activated cells were seeded into PHA-containing agar a straight line was obtained (Fig 6.14B; see also Fig 3.3) which did not intersect the X-axis near the origin. A threshold number of cells seemed to be required for colony production in the presence of PHA but not with IL2. The results from 7 independent experiments, summarised in Table 6.4, are consistent with these findings. If the responding cells (colony forming cells) are considered as "kinetic particles" the requirement for cellular interactions may be mathematically analysed by the method originally described by Coppleston and Michie (1966) and further evaluated by Kondracki and Milgrom (1977) and utilised by a number of workers (Mosier and Coppleston, 1968; Goube de Laforest et al., 1979b; Tse, Schwartz and Paul, 1980; Gelfand et al., 1981). The principle behind this test is that the number of cell interactions (i.e. populations) required to produce a measurable event (colony) is obtained from the slope of the regression line when the logarithm of lymphocyte concentration is plotted against the logarithm of the response (number of colonies). Theoretically the slope of the regression line indicates the order of kinetics, which reflects the number of "kinetic particles" and therefore the number of cell

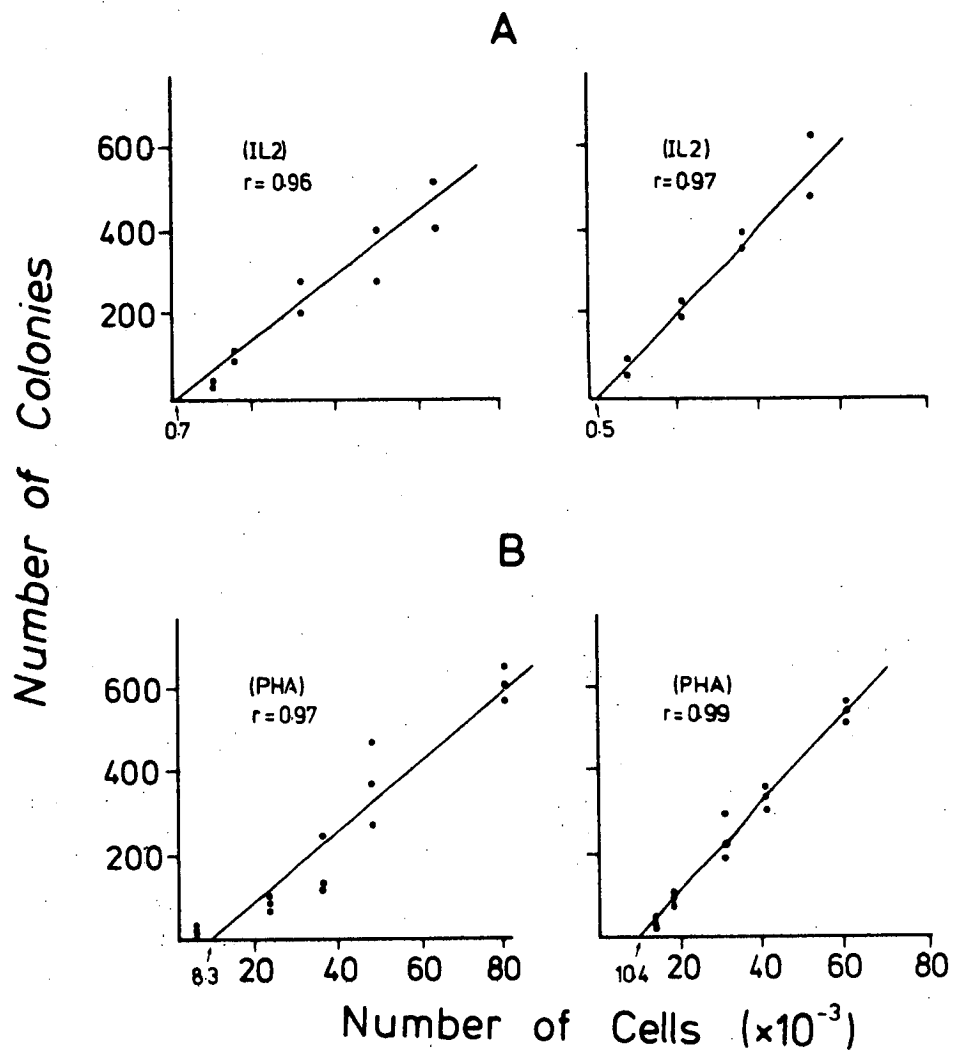


Fig 6.14(A and B)

Relationship between number of colonies and number of cells seeded for PHA-treated cells seeded into either IL2-containing agar (Fig 6.14A) or PHA-containing agar (Fig 6.14B). Each point represents the number of colonies for each of the replicate cultures. Results from two representative experiments for cells seeded into IL2 or PHA. r = correlation coefficient.

interactions required to produce one measurable event (colony). The slope of the log/log curve will be governed by the shape, slope and X-axis intercept of the corresponding linear dose-response curve. When the data from Fig 6.14 were transformed into their log values and replotted (Fig 6.15) the resulting regression lines, reflecting the number of interacting cells, had values of 1.1 and 1.9 for cells cultured in the presence of IL2 and PHA respectively. (The slopes of the regression lines from the 7 independent experiments were 1.2 ± 0.1 and 1.8 ± 0.1 (Table 6.4) for cells cultured in the presence of IL2 and PHA respectively.)

6.3.6 Colony growth from PHA or IL2 pulsed cells

To obtain further information concerning the roles of IL2 and PHA in colony formation mononuclear cells (PHA-treated or freshly isolated) were exposed to PHA for varying time intervals prior to seeding into agar medium which contained either PHA or IL2. The results in Fig 6.16 demonstrate colony production when cells, exposed to PHA for a minimum of 1 h, were seeded into IL2-containing agar; similar cells seeded into PHA-containing agar required a preincubation culture of 20 h to produce optimal growth. In addition, unstimulated cells seeded directly into agar medium containing both PHA and IL2 developed into colonies, demonstrating that the preincubation culture was not essential provided an adequate supply of IL2 was available. When PHA-treated cells were pulsed with either PHA or IL2 for 1 h immediately prior to seeding into agar which contained neither PHA nor IL2, there was no colony growth (Fig 6.16).

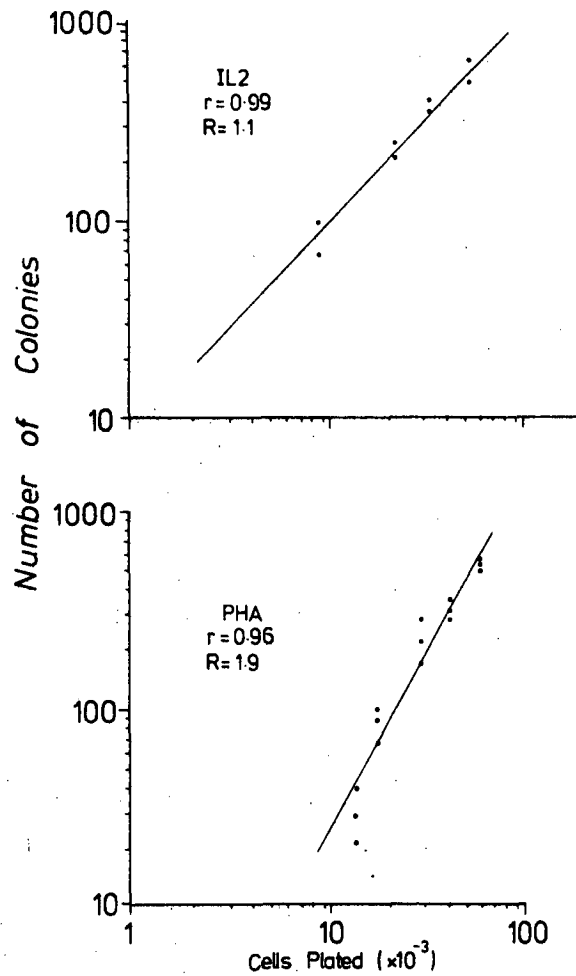


Fig 6.15

Relationship between number of colonies and number of cells seeded (from Fig 6.14). Replotted as log/log cell dose response curves for PHA-treated cells seeded into either IL2-containing agar (top) or PHA-containing agar (bottom). Each point represents the number of colonies for each of the replicate cultures. r = correlation coefficient, R = regression coefficient.

Table 6.4

Summary of the important features of the cell dose response curves when PHA-treated cells were seeded into PHA- or IL2-containing agar

liquid	agar	correlation coefficient	X-axis intercept	log/log slope
PHA	PHA*	0.96 ± 0.01	9.4 ± 0.6	1.8 ± 0.1
PHA	IL2'	0.97 ± 0.003	1.5 ± 0.5	1.2 ± 0.1

Results represent the mean \pm the standard deviation of the mean from replicate experiments

* Results from 4 experiments

' Results from 3 experiments

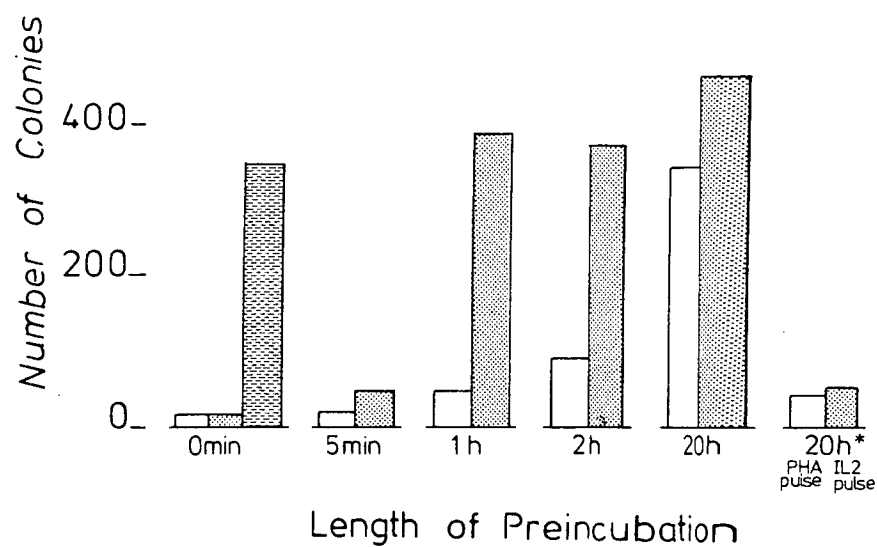


Fig 6.16

Effects of exposing cells to PHA for varying time intervals prior to seeding into agar containing either PHA (□) or IL2 (▨) or directly into PHA and IL2 (▤). * Cells were preincubated with PHA for 20 h then pulsed with fresh PHA or IL2 for 1 h after which time they were seeded into agar medium from which both PHA and IL2 were omitted (see 6.2.4). Columns are the mean number of colonies from duplicate plates. A representative experiment is shown.

6.3.7 Production of colonies from adherent cell depleted populations

It was shown in section 5 that cell populations depleted of adherent cells prior to the preincubation culture did not form colonies when seeded into PHA-containing agar. If these same PHA-stimulated non-adherent cells were seeded into IL2-containing agar colony growth was observed (Table 6.5).

6.3.8 Lack of colony growth in the presence of cytochalasin B

Preliminary studies investigating the involvement of the microfilament/microtubule cytoskeletal control system of surface membrane receptors were performed with the microfilament disrupting agent cytochalasin B (CB). CB was added to the cultures at either the activation ($T \rightarrow T'$) or proliferation ($T' \rightarrow nT'$) phase.

6.3.8.1 Treatment during the preincubation ($T \rightarrow T'$) phase

Cells treated with CB and PHA did not produce colonies when they were seeded into IL2-containing agar (Table 6.6).

6.3.8.2 Treatment during the proliferation ($T' \rightarrow nT'$) phase

Results from Table 6.6 also illustrate that PHA-activated cells cultured in the presence of both IL2 and CB did not form colonies.

6.3.9 Requirement for SRBC

When PHA-activated cells were seeded into IL2-containing agar, in the absence of SRBC, no colony growth resulted (Table 6.7).

Table 6.5

Colony growth of non-adherent cells (NAC), treated with PHA, then seeded into either PHA- or IL2-containing agar

Cell Population	(liquid)	(agar)	Number of colonies*			
			Exp. 1		Exp. 2	
MN	PHA	PHA	160	195	250	320
NAC	PHA	PHA	6	15	2	17
MN	PHA	IL2	225	210	340	295
NAC	PHA	IL2	150	198	225	190

* Number of colonies per 50 000 PHA-treated cells seeded into IL2- or PHA-containing agar. Results represent the number of colonies produced from the duplicate plates of both experiments.

MN unseparated mononuclear cells

NAC mononuclear cells depleted of adherent cells prior to the liquid preincubation culture

Table 6.6

Colony forming ability of cells treated with cytochalasin B during either the preincubation or proliferative culture

EXP.NO	(liquid) (agar)	Number of colonies*					

		PHA IL2		PHA + CB IL2		PHA IL2 + CB	
1		260	280	0	0	0	0
2		230	320	0	0	0	0

* Number of colonies produced per 50 000 PHA-treated cells seeded into IL2-containing agar from duplicate plates for each experiment.

CB 10 ug/ml cytochalasin B

Table 6.7

Requirement of colony formation for sheep-RBC when
PHA-treated cells were seeded into IL2-containing agar

	Number of colonies*			
	Exp. 1		Exp. 2	
WITH SRBC	160	195	250	320
WITHOUT SRBC	6	15	2	17

*Number of colonies produced per 50 000 PHA-treated
cells from duplicate plates for each experiment.

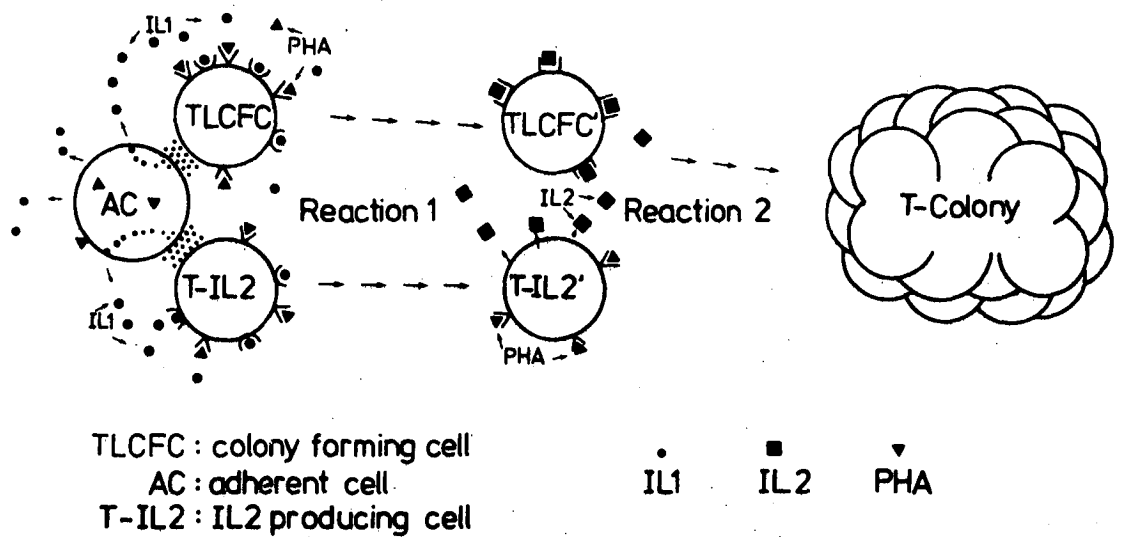


Fig 6.17

Model for the development of PHA-induced human T lymphocyte colonies in semisolid agar involving two reactions, three signals and three interacting cell populations. Reaction 1 ($T \rightarrow T'$) is driven by two signals. PHA binds to both colony forming cells (TLCFC) and IL2-producing cells providing one signal while adherent cells, through cell-cell contact (encouraged by PHA), produce the other (IL1-mediated) signal and as a result the relevant cells become activated. These activated cells either release IL2 on further exposure to PHA or proliferate into colonies in response to IL2 which provides the third and final signal for colony formation ($T' \rightarrow nT'$) and constitutes reaction 2.

6.4 DISCUSSION

6.4.1 Colony promoting activity of IL2

T lymphocyte colonies developed when PHA-treated lymphocytes were cultured in the presence of IL2 (Fig 6.1; Table 6.1). This is in agreement with the results from established liquid culture systems demonstrating that activated lymphocytes proliferate in the presence of IL2 (Morgan, Ruscetti and Gallo, 1976; Gillis and Smith, 1977; Gillis et al., 1978; Rosenberg et al., 1978; Watson, 1979; Woolnough and Lafferty, 1979). As shown in Fig 6.6 colony formation responded to IL2 in a dose-dependent manner and the colonies were generally larger and more numerous than those produced in the presence of PHA (Fig 6.1; Table 6.1) suggesting that the IL2 preparation was more efficient in promoting the proliferation of activated lymphocytes. The greater efficiency of the IL2 preparation could be explained by a number of factors. The most likely being that the IL2 preparation, which will contain high concentrations of IL2, can be incorporated into the cultures at optimally high concentrations whereas colony growth within the PHA system will rely on the in situ synthesis and release of IL2 from the IL2-producing cells and optimum concentrations of IL2 may not be produced. Alternatively (or in addition) PHA might not only stimulate the release of colony enhancing factors (IL2) but other factors that may have negative or suppressive effects. Consequently, colony growth in the presence of PHA will be governed by the interactions of colony forming cells with cooperating cells and their products. The effectiveness of these interactions will determine the colony forming capacity.

The augmentation of colony formation by IL2 did not correspond to a similar increase in either the number of clusters

(Fig 6.2) or the number of colonies plus clusters combined (Fig 6.3). This implies the improved colony forming conditions brought about by IL2 were due to an overall increase in the number of cell divisions of the already dividing cells (i.e. those cells that possess the ability to produce clusters or colonies in response to PHA). These additional divisions will a) enable some clusters to be converted into colonies thereby increasing the colony frequency and b) enable the colony forming cells to produce larger colonies.

When both PHA and IL2 were incorporated throughout the proliferative phase of colony growth the number of colonies was not significantly greater than in the presence of IL2 alone (Fig 6.8). This was also observed when clusters (Fig 6.9) or colonies plus clusters (Fig 6.10) were considered. Thus IL2 and PHA did not amplify each other's action when they are both present during the agar culture. This suggests that they exert their ultimate effect on the same target (proliferating) cells but, as discussed later, they follow different pathways.

Inclusion of both PHA and IL2 during the preincubation (activation) culture did result in enhanced colony growth when the treated cells were seeded into either PHA- or IL2-containing agar (Fig 6.11). Again, this enhanced colony growth did not correspond to a similar increase in the number of clusters (Fig 6.12) or the combined total of colonies plus clusters (Fig 6.13). The inability of these improved culture conditions to stimulate the replication of non-cluster and non-colony forming lymphocytes implies that it is unlikely that all PHA-treated cells proliferate when seeded into agar medium.

On the occasions that PHA was unable to convert PHA-treated cells into colonies there was good colony growth when IL2 was incorporated during the agar culture (Table 6.1; Fig 6.1). This would suggest the lack of colony growth within the PHA system was due to a defect in IL2 production rather than a defect in the colony forming cell itself. It is unlikely that PHA non-responsiveness was due to the production of inhibitory proteins because, as shown in Fig 6.8, when both PHA and IL2 were added together during the agar phase there was normal colony growth even though this individual was a PHA non-responder. This non-responsiveness in colony formation has been previously reported (Riou et al., 1976; Zeevi, Goldman and Rozenszajn, 1977; Dao et al., 1978; Klein et al., 1981a) and the lack of colony growth on these occasions might be due to IL2 production defects.

6.4.2 Cellular interactions and colony formation

Most authors agree that T colony formation requires an interaction of colony forming cells with a heterogeneous population of cooperating cells (Claesson et al., 1977b; Zeevi et al., 1977; Spitzer et al., 1980; Rozenszajn et al., 1981; Klein et al., 1982). The nature of these cooperating cells is controversial as T cells (Zeevi et al., 1977), B cells (Klein et al., 1982) or T and B cells (Spitzer et al., 1980) have been proposed. Ascertaining the nature of these interacting cells would require precise cell separation and reconstitution experiments. However an indirect approach can be pursued by analysing the logarithm of the number of cultured cells against the logarithm of the response. The slope of this log/log curve, which is a straight line, indicates the minimum number of interacting cell populations required to produce the response (Kondracki and Milgrom, 1977; Tse et al., 1980). Such an analysis has been performed by Goube de

Laforest et al. (1979b) and Gelfand et al. (1981) who obtained slopes of 3 and 2 respectively. This discrepancy is most likely due to the different culture conditions. Although both these workers employed single step culture techniques Gelfand et al. (1981) utilised conditioned medium and the saturation of growth factors within this medium may have replaced the need for at least one of the cell populations. Goube de Laforest et al. (1979b) proposed that monocytes and T cells cooperated with the colony progenitor cells to allow colony production. On the other hand Gelfand et al. (1981) tentatively proposed colony formation required colony progenitor cells and a population of accessory T cells. A number of reports support the view that T cells "help" other T cells to form colonies (Zeevi et al., 1977; Spitzer et al., 1980; Rozenszajn et al., 1981). There have been contradictory reports regarding the role of monocytes. Some workers have reported monocytes as essential for colony growth (Claesson et al., 1977b; Rozenszajn et al., 1981; section 5) but others have implicated monocytes as suppressive and therefore unnecessary (Zeevi et al., 1977; Klein et al., 1982). It has become increasingly evident that monocytes are a heterogenous population of cells consisting of helper and suppressor monocytes and it is likely they exert dual effects on colony formation according to their concentration (Rozenszajn et al., 1981). At small concentrations they are required for the early activation events but, when in larger concentrations, or in a highly stimulated state they may release prostaglandins (Bockman and Rothschild, 1979) and other inhibitory proteins (Sredni et al., 1978).

A log/log mathematical analysis of cell number response curves was carried out in the present work. By this means it was shown

that, as a slope of 2 was obtained, T colony formation was the result of interactions between at least two cell types when PHA-treated cells were seeded into PHA-containing agar (Table 6.4). It must be realised that as a two step procedure was used the cellular interactions involved in activation are not part of this analysis because the cells seeded were already activated. In section 5 it was shown that adherent cells were essential for this activation process. Therefore T colony formation is the result of interactions between at least 3 different cell populations. The requirement for 3 cooperating cell populations compares favourably to the cellular interactions described for cytotoxic T cell responses (Lafferty et al., 1980; Ruscetti and Gallo, 1981; Smith and Ruscetti, 1981) suggesting the underlying mechanisms for colony growth and cytotoxic T cell responses are similar. A prediction from this comparison is that the third cooperating cell population sustains colony growth through the production of IL2 and is therefore likely to be an activated T cell. This is supported by the results shown in Fig 6.15 and Table 6.4 as the log/log slopes were reduced to approximately 1 when IL2 replaced PHA during the proliferative culture. Further support for this idea can be derived from Fig 6.14. The linear curve of cells versus colonies, in the presence of PHA, did not cross the X-axis near the origin suggesting a build-up of accessory cells must occur before colonies develop and that cellular interactions are required. The linear curve in the presence of IL2 intersected the X-axis near the origin indicating that IL2 replaced the need for this build-up of accessory cells. This replacement of the cooperating cells by IL2 suggests the accessory cells were IL2 producing cells (activated T cells) and the role of PHA was to stimulate these cells to release IL2 which in turn maintained

the proliferation of the activated colony forming cells.

Further insight into the events involved in T colony development can be obtained from the pulse exposure experiments outlined in Fig 6.16. Cells pulsed for one hour and seeded into IL2-containing agar produced optimal colony growth whereas a 20 h incubation was required when these PHA-treated cells were seeded into PHA-containing agar. This difference suggests a lack of IL2 production when the cells were pulsed with PHA for one hour prior to seeding into PHA-containing agar. As activation of the IL2-producing cells is a two signal process with antigen or mitogen providing one signal and IL1 the other (Lafferty et al., 1980) it is likely that this costimulator activity was deficient in the PHA pulse system. With this in mind the 20 h preincubation prior to seeding into PHA-containing agar would be necessary for an effective expression of the costimulator activity. This would enable the activation of IL2-producing cells to release IL2 on further exposure to PHA. Therefore the preincubation culture facilitates the production of IL2 through the combined action of IL1 and PHA. Others have proposed that lymphocytes stimulate monocytes to release IL1 via a cell-contact (Ir gene; Ia antigen recognition) mechanism (Unanue, 1980; Oppenheim and Gery, 1982). The results reported herein are consistent with these findings as the liquid preincubation culture, through the leuco-agglutinating properties of PHA, will favour the vital cell-cell contact requirement. A short pulse with PHA will be sufficient to allow binding of PHA while the release of IL1 proteins in response to cellular contact and through Ia antigen recognition will require a longer incubation. If cells, pulsed with PHA, are cultured in PHA-containing agar no further activation of the IL2 producing cells (and therefore IL2 production) will occur due

to prevention of cell contact by the high agar concentration. However, if these cells were cultured in IL2-containing agar colony growth would proceed because the colony forming cells have received a signal from PHA during the brief exposure period and will be further activated by the IL2 preparation resulting in cell division and colony formation. Although the activation of the IL2 producing cells has been established as a two signal process (Smith and Ruscetti, 1981) there are contradictory reports as to the mechanism of the activation of the proliferating cells. Some reports suggest that activation occurs in response to mitogen alone (Smith and Ruscetti, 1981) whereas others have suggested that IL1 activity is also essential (Lafferty et al., 1980). The results reported herein tend to support the proposal that mitogen alone is sufficient to activate colony forming cells because the minimum requirements for colony formation are the binding of PHA to the colony forming cells and a source of IL2 activity. However the two signal model of activation is not refuted by these results due to possible presence of IL1 activity in the IL2 preparation (see 6.4.4). When cells were pulsed with PHA and seeded into IL2-containing agar it was possible that colony forming cells were rendered responsive to IL2 by the IL1 activity which resided in the IL2 preparation. As such, the colony forming cells would receive the first signal from PHA in the liquid culture and the second (costimulator) signal by the IL1 residing proteins during the agar culture. Perhaps the mechanisms of mitogen and antigen stimulation follow slightly different pathways? To answer this question absolutely pure interleukin preparations and cell populations will be necessary.

Cell populations depleted of adherent cells will not multiply on exposure to PHA, presumably due to a deficiency in IL1 or

costimulator activity. This is shown in Table 6.5 when NAC's treated with PHA failed to form colonies when they were cultured in the presence of PHA. However the same population of cells seeded into agar supplemented with the IL2 preparation were able to produce colonies. As with the pulsing experiments the need for monocytes could be bypassed with the IL2 preparation.

6.4.3 Colony growth requires a continual presence of IL2

Cells activated by PHA did not form colonies unless either IL2 or PHA was present throughout the agar culture (Table 6.1; Fig 6.16). It has been shown that activated T cells absorb IL2 from the surrounding culture medium (Gillis et al., 1978; Coutinho, et al., 1979; Smith and Ruscetti, 1981) and, as daughter T cells will also express IL2 receptors (Smith and Ruscetti, 1981), a constant source of IL2 will be necessary to maintain proliferation. The need for PHA throughout the proliferative (agar) culture has been described in a number of T colony papers (Rozenszajn et al., 1975; Foa and Catovsky, 1979) and, although activated T cells release IL2 after a single exposure to antigen, the continued presence of PHA was necessary as one exposure would not produce sufficient IL2 for the entire culture period. PHA (and presumably antigen) therefore stimulates the release of IL2 but once this stimulus was removed the production of IL2 was limited and the T cell response can be regulated at this level. On occasions, as revealed in Table 6.1, activated cells may form colonies when seeded into agar medium lacking either PHA or IL2. The most likely explanation for this observation is, not that cells can divide without an additional stimulus, but that lymphocytes from certain individuals may, in the absence of a further stimulus, release

sufficient quantities of IL2 to enable cell division and therefore colony production.

Although IL2 replaced the second or cooperating cell during the proliferative phase and generally improve the overall plating efficiency it was unable to replace the need for SRBC (Table 6.7). This suggests the IL2 preparation does not contain the factor(s) secreted by the red cells (section 4).

From these results a general scheme for T colony growth can be formulated and is illustrated in Fig 6.17. As with cytotoxic T cells the activation and proliferation of TLCFC's is a two step, three signal process. The first step (activation) is driven by two signals. One of these signals is mediated through the binding of PHA to both TLCFC's and IL2-producing cells while the other signal (IL1) is presented to these cells by adherent cells (monocytes) in response to PHA (antigen) and/or Ia antigen recognition through cell-cell contact. As a result of these two signals the relevant cells become activated and either a) release IL2 on further exposure to PHA or b) proliferate to form colonies in the presence of IL2, which is the third and final signal for the complete T cell proliferative response. Regulation of T colony formation can occur at (at least) two levels. Cellular activation requires the presence of PHA (antigen) while proliferation of these activated cells will only occur in the presence of IL2. As IL2 is released from activated cells in response to PHA then removal of PHA from the system will regulate the response through a lack of IL2 production. The other level of regulation is through monocytes and their products. At low concentrations they are essential for the production of IL1 but in higher concentrations the monocytes may release inhibitory proteins that block colony formation. PHA in this

system acts at three levels; it is important for the activation of 1) TLCFC's and 2) IL2-producing cells and it also is responsible for 3) the release of IL2 from activated IL2-producing cells. This scheme, although consistent with liquid culture systems, is in contrast with the tentative scheme proposed by Klein et al. (1982). In their model they propose that monocytes act at a purely suppressive level whereas the colony promoting activity is provided by B cells rather than activated T cells. The most likely explanation for the differences between these two models could be that different colony assay systems were used. Within the two step system it is more feasible to study the individual reactions of T cell responses, with particular reference to monocyte function, than the single step system of Klein et al. (1982). Although B cells may have colony promoting activity it is more likely that activated IL2-producing T cells are intimately involved in providing help for the activated colony precursor cells.

6.4.4 Non-IL2 activities of the lymphokine preparation

The action of IL2 in improving colony formation when added in combination with PHA during the preincubation culture suggested that the IL2 activities of the IL2 preparation were not entirely restricted to the maintenance of activated cells. As some IL2-preparations have been shown to support thymocyte proliferation (Oppenheim and Gery, 1982), a definitive but not specific bioassay for IL1, it is possible that IL2-preparations may on specific occasions mimic IL1 by promoting T cell activation. The most probable explanation for this apparent IL1 activity is that the IL2 preparation contained IL1 proteins because a) the IL2 was prepared from tonsil mononuclear cells which were likely to include a number of IL1 producing cells (monocytes) and b) IL1 is necessary for IL2 production

(Smith et al., 1980). The promotion of activation may therefore be due to the presence of IL1 proteins. Alternatively the presence of both IL2 and PHA during the activation phase may improve colony production by making the system more efficient as the exogenous IL2 would bind to the cells immediately the IL2 receptors were expressed.

Further evidence for an activity of this IL2 preparation at early activation stages can be derived from Table 6.2 where 4/10 cultures formed colonies after preincubation with IL2 and seeding into PHA-containing agar. In the appropriate control experiments when cells were seeded into PHA-containing agar after preincubation with medium alone there was no colony growth. In the 6/10 experiments (Table 6.2) in which preincubation with IL2 alone did not lead to colony growth the concentration of PHA was not the critical factor (Fig 6.4). The apparent ability of the IL2 preparation to participate in T cell activation was not due to contaminating PHA as the IL2 preparation on its own was unable to initiate and support blast transformation and is therefore non-mitogenic (Fig 6.5). In addition good colony growth from IL2 treated cell suspensions only occurred if the cells were seeded into agar medium containing PHA. If the cells were cultured with IL2 there was only minimal colony growth (Table 6.3). This suggested that for an IL2 activity to be expressed during the early stages of T colony growth a second antigen- or mitogen-mediated signal was required. As cells responded to IL2 during the activation phase in only 4/10 cultures it is possible that a suboptimal level of IL1 was present in the IL2 preparation. If this was true then the results from Table 6.2 provide evidence for the possibility that T cell activation in response to PHA (antigen) and IL1 is independent of a specified order of binding.

Larsson, Andersson and Coutinho (1978) suggested that IL2 may support the proliferation of in vivo activated lymphocytes and therefore in vitro T cell expansion may not, on occasions, require initial antigen or mitogen binding for stimulation. This finding was contradicted by Warren and Pembrey (1981) who claimed that IL2 was unlikely to be responsible for the maintenance of in vivo activated cells because titration end points for maintenance and mitogenic activities were different. The results described in Table 6.3 support Warren and Pembrey as IL2 in the absence of PHA was unable to support colony growth. This indicates that PHA exposure was a crucial factor and that a maintenance of in vivo activated lymphocytes can not account for the apparent IL2 activity during early activation events. An alternative explanation for the early IL2 activity could be that IL2 binds to activated lymphocytes as well as a subpopulation of resting lymphocytes. This is unlikely because, as shown through absorption studies, T lymphocytes do not express receptors for IL2 whilst in a resting or quiescent state (Gillis et al., 1978; Bonnard, Yasaka and Jacobson, 1979; Coutinho et al., 1979; Smith et al., 1979; Watson and Mochizuki, 1980). The binding of IL2 to lymphocytes is restricted to activated cells. Therefore the most likely explanation for the apparent IL2 activity during the early activation phase is due to the presence of IL1 proteins in the IL2 preparation.

6.4.5 Involvement of the microfilament network in colony formation

Very little is known how messages, received at the surface of the cell's membrane, are transmitted to the nucleus to induce the nuclear replicatory process. It is interesting to hypothesise that internalisation of membrane receptors and their complexes is an

important part of this process and that the surface membrane binding can be modelled along the lines of hormone-receptor systems. In an interesting comparative study Das (1982) performed a number of experiments to probe the mechanism of action of epidermal growth factor (EGF) which was described as a "prototypical mitogenic hormone". It was concluded that internalisation of the EGF-receptor complex was essential if early cellular activation events were to occur and that intracellular signals for mitogenesis were similar for all types of growing cells.

Resting mononuclear cells express surface membrane receptors for a wide range of molecules including proteins and glycoproteins (Hobart and McConnell, 1976). Activated T cells can be distinguished from resting T cells because they express receptors for IL2 (Bonnard et al., 1979; Coutinho et al., 1979; Smith et al., 1979; Ruscetti and Gallo, 1981). Studies with fluorescently labelled mitogens (eg FITC-Con A) have shown that receptor-complexes are initially distributed randomly throughout the cell membrane (Oliver, Zurier and Berlin, 1975; Nicolson, 1976; Schreiner and Unanue, 1976). The complexes are reorganised to form patches or caps. Following this reorganisation the entire soluble complexes are internalised. The process of receptor redistribution and capping is an active process requiring an intact and complicated cytoskeletal network of microfilaments and microtubules (Nicolson, 1976). As a consequence of microfilament disruption the control of membrane receptors, governed by this cytoskeletal network, will be disorganised and therefore the control of receptor binding complexes and possible internalisation will be affected. The results from the experiments with CB, although only preliminary, suggest a role for this network at both

activation and proliferation stages (Table 6.6). It is interesting to speculate that both PHA (and antigen) and IL2 exert their effects through the processes of capping and internalisation of their receptor-complexes. To ascertain how PHA (antigen), IL1 and IL2 exert their effects experiments based on knowledge gained from studies with hormone-receptor complex interactions should be performed.

6.5

SUMMARY

A number of contrasting proposals have been suggested concerning the roles and identities of cooperating cells involved with human T lymphocyte colony formation. For this reason studies using a lymphokine with IL2 activity were initiated and indirect mathematical analyses were performed to determine the events involved in the formation of T lymphocyte colonies. It was shown that T colony development could be outlined by the two interdependent reactions described in Fig 6.17 of activation ($T \rightarrow T'$) and proliferation ($T' \rightarrow nT'$). In these reactions the cooperation of three cell populations was essential. The first reaction required adherent cells (section 5), which together with PHA, provided the necessary signals for activation. Cell contact was essential to allow the expression of this adherent cell or IL1 activity. As a result of these signals at least two subpopulations of lymphocytes were activated, the T lymphocyte colony forming cells themselves and a population of cooperating T cells. These activated T cells interacted during the second or proliferative reaction. In response to PHA the cooperating T cells released IL2 thereby enabling the activated TLCFC's to proliferate into colonies. To maintain proliferation a continued supply of IL2 was essential, this required constant stimulation of the activated IL2-producing cells by PHA.

Incorporation of cytochalasin B at either the activation or proliferation phase inhibited colony growth suggesting that the microfilament microtubule network was an important feature of T cell responses with respect to redistribution of membrane - receptor complexes.

S E C T I O N S E V E N

THE EFFECTS OF LOW DOSE IRRADIATION
ON T LYMPHOCYTE COLONY FORMATION

The lethal effects of irradiation are usually expressed by mitotically active cells (Ehmann et al., 1974) while non-dividing cells are relatively resistant to doses of irradiation that can kill their dividing counterparts (Alper, 1979). Lymphocytes belong to a unique population of cells because they can be killed by low irradiation doses without undertaking cell mitosis and therefore suffer interphase death (Anderson and Warner, 1976). Many studies have concentrated on this feature of lymphocyte death and various parameters such as viability, rosette formation and morphological aberrations have been utilized to assess the extent of radiation damage (Schrek, 1955; Schrek, 1959; Bari and Sorenson, 1964; Stefani and Schrek, 1964; Jordon, 1967; Stefani, Chandra and Tonaki, 1977; Kwan and Norman, 1977). These methods do not measure proliferative or functional properties and studies of the effects of irradiation on cycling or dividing lymphocytes have been limited to assays of tritiated thymidine uptake (Circovic, 1969; Sato and Sakka, 1970; Rickinson and Ilbery, 1971; Herva and Kiviniitty, 1975; Baral and Blomgren, 1976; Fauci, Pratt and Whalen, 1978). These studies have a limited application since irradiation itself, in the absence of antigen or mitogen, may stimulate the incorporation of tritiated thymidine into DNA (Evans and Norman, 1968). In addition a delay in mitosis was reported in response to low irradiation doses and the length of the culture period after irradiation was found to be critical as 72 h cultures produced larger effects than 48 h cultures (Rickinson and Ilbery, 1971). Other workers have provided evidence that lymphocytes can differentiate into blast cells after irradiation

but these blast cells have a limited mitotic ability (Conard, 1969; Sato and Sakka, 1970; Rickinson and Ilbery, 1971; Stewart and Perez, 1976). Thus, these studies do not reliably assess the effects of irradiation on dividing lymphocytes.

The conventional method for measuring the effects of irradiation on dividing cells is to assay for mitotic (or reproductive) death. In radiobiological terms mitotic death is defined as the inability of a cell to give rise to daughter cells. To assay for mitotic death the ability of cells to produce colonies of daughter cells after exposure to various doses of irradiation is determined. A colony forming assay for mitotic death was first described for yeast cells by Puck and Marcus (1956) who analysed the results with a survival curve. The parameters of this curve, specifically the D_0 value and the extrapolation number (n), provide convenient measurements of radiation effects on the cell population (appendix I). The assay is limited to cells capable of colony formation, and has not hitherto been applied to lymphocytes.

By inducing colony formation in lymphocytes an increased radioprotection might be expected as protection from irradiation by PHA stimulation had been demonstrated with many non-dividing lymphocyte functions (Schrek and Stefani, 1964; Stefani, 1966; Conard, 1969; Rickinson and Ilbery, 1971; Anderson and Warner, 1976). It is likely that the increased metabolic activity associated with mitogen stimulation corresponded to this increased radioprotection. The aims of this section were to evaluate the possible radioprotective effect of mitogen stimulation on lymphocyte cell division and to ascertain the radiosensitivity of dividing lymphocytes in an agar culture system. Lymphocytes were irradiated at different stages of their

activation to assess what contribution PHA-stimulation might have on survival of the cells. Lymphocytes were irradiated whilst in a resting state (i.e. before PHA exposure) or in an active metabolic state (i.e. after PHA exposure) and colony formation was analysed with the conventional survival curve parameters. Appendix I provides a general background to the field of radiobiology and Appendix II contains the dose response curves for colony formation from all the replicate experiments described throughout this section.

7.2 METHODS

7.2.1 Irradiation

Irradiation was performed in either polystyrene culture tubes or polystyrene culture dishes. All of the irradiations were performed at room temperature by an Ultrays 140 kV mA X-ray machine delivering a dose of 0.68 Gy/min at the anode to target distance of 25 mm and Cu/Al filtration. Irradiated and sham irradiated cultures were returned to the incubator within 30 min of being removed. Sham irradiation was performed by transporting the cultures to and from the X-ray machine without exposing them to any radiation.

7.2.2 Cell recoveries, viabilities and E rosette enumeration

Total cell counts, viabilities and E rosettes were calculated after all liquid preincubation cultures according to the techniques outlined in sections 2.2.2 & 3.2.3

7.2.3 Irradiation protocol

7.2.3.1 Irradiation of resting lymphocytes:

a) 24 h before exposure to PHA

Mononuclear cells at a final concentration of one million

per ml were suspended in sterile culture tubes in 1 ml of alpha medium supplemented with 5% FCS and irradiated with doses ranging from 0-4 Gy. After irradiation the cultures were incubated for 24 h at 37°C in a humidified atmosphere of 10% CO₂ in air. Following this initial incubation 10 ug of PHA was added to all cultures and they were incubated for a further 20 h after which time the cells were washed and seeded into PHA-containing agar at a final concentration of 50×10^3 cells/ml. After 7 days the number of colonies and clusters was counted. Triplicate plates were cultured and each experiment was performed in quadruplicate. Scheme A of Fig 7.A.

7.2.3.2 Irradiation of resting lymphocytes:

b) immediately prior to PHA exposure.

Mononuclear cells at a final concentration of 1×10^6 /ml were suspended in sterile culture tubes in 1 ml of alpha medium supplemented with 5% FCS and irradiated with doses ranging from 0-4 Gy. Immediately after the irradiation 10 ug of PHA was added to each culture and they were incubated for 20 h after which time the cells were washed and seeded into PHA-containing agar at a final concentration of 50×10^3 cells/ml. The plates were assayed after 7 days for colony and cluster numbers. Triplicate plates were cultured and each experiment was performed in quadruplicate. Scheme B of Fig 7.A.

7.2.3.3 Irradiation of activated lymphocytes:

c) 20 h after exposure to PHA

In preliminary experiments 20 h PHA treated cells were washed and resuspended in polystyrene culture tubes at a final concentration of 1×10^6 /ml. These cells were irradiated, washed and

seeded into PHA-containing agar. Alternatively mononuclear cells were suspended at a final concentration of one million cells per ml in alpha medium supplemented with 5% FCS and 10 ug PHA and incubated for 20 h after which time the cells were washed and seeded into PHA-containing agar at a final concentration of 50×10^3 cells/ml. The irradiation was immediately performed in the petri dishes with each plate receiving a dose within the range of 0-4 Gy. As the original preparation of 1×10^6 cells would not yield enough cells for all the necessary agar cultures at least 3 cultures were incubated and the PHA-treated cells were pooled prior to seeding into the agar. The plates were assayed after 7 days for colony and cluster numbers and triplicate plates were cultured with each experiment performed in quadruplicate. Scheme C of Fig 7.A.

7.2.3.4 Irradiation of activated cells:

d) 3 days after exposure to PHA

Mononuclear cells at a final concentration of 1×10^6 /ml were suspended in 1 ml of alpha medium supplemented with 5% FCS and 10 ug PHA in sterile culture tubes and incubated for 20 h in a humidified atmosphere of 10% CO₂ in air after which time the PHA treated cells were washed and seeded into PHA-containing agar at a final concentration of 50×10^3 cells/ml. These agar cultures were incubated for 2 days before they were irradiated within the range of 0-4 Gy. Following irradiation the plates were incubated for a further 5 days and scored for colony and cluster numbers. For all irradiation doses triplicate plates were performed and each experiment was performed in quadruplicate. Scheme D of Fig 7.A.

Fig 7.A

Schemes used to incorporate the irradiation procedures into the colony forming technique.

Scheme A) Irradiation 24 h prior to PHA exposure:-

Mononuclear cells were irradiated in liquid culture. These cells were then incubated for 24 h after which time PHA was added and the cultures were incubated for a further 20 h prior to seeding into agar medium.

Scheme B) Irradiation immediately prior to PHA exposure:-

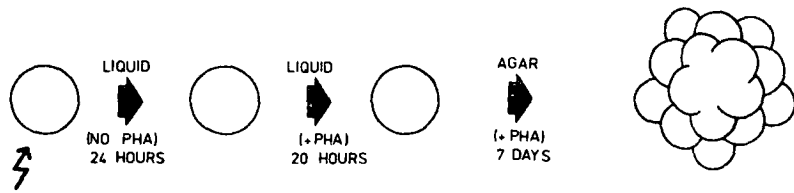
Mononuclear cells were irradiated in liquid culture. Immediately following the irradiation PHA was added and the cells were incubated for 20 h prior to seeding into agar medium.

Scheme C) Irradiation 20 h after PHA exposure:- Mononuclear

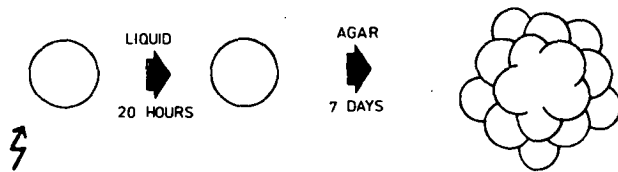
cells were incubated with PHA for 20 h prior to seeding into agar medium. Immediately after the cells were seeded into agar the cultures were irradiated.

Scheme D) Irradiation 3 days after PHA exposure:-

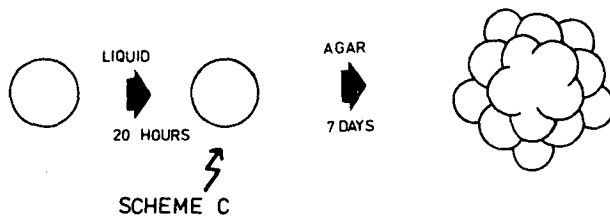
Mononuclear cells were incubated with PHA for 20 h prior to seeding into agar medium. The agar cultures were incubated for 2 days prior to irradiation.



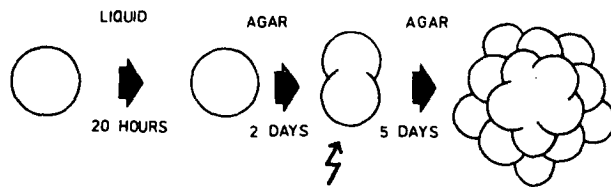
SCHEME A



SCHEME B



SCHEME C



SCHEME D

7.2.4 Analysis of results

7.2.4.1 Colony growth and surviving fractions

All cultures (excluding those irradiated 24 h before PHA stimulation) were cultured for 20 h in liquid phase and 7 days in semisolid phase. At the termination of the agar cultures the number of colonies and clusters was scored according to the criteria outlined in section 2.2.4.

The arithmetic means of the colony and cluster counts were determined for the triplicate plates of each irradiation dose. Surviving fractions were calculated by expressing the mean colony or cluster counts for each irradiation dose as a fraction of the mean colony or cluster count of the control (sham irradiated) plates. Therefore the surviving fraction for colony growth after irradiation =

$$\frac{\text{mean colony count of irradiated cultures}}{\text{mean colony count of unirradiated cultures}}$$

7.2.4.2 Dose response and survival curves

The dose dependent inhibition of colony growth was plotted as a dose response curve on linear axes. Each experiment was graphed separately and the total number of colonies was plotted against the irradiation dose (all the replicate curves are displayed in appendix II while single representative curves for each irradiation protocol are shown in Fig 7.1).

For the survival curves, the logarithm of the surviving fraction from all of the four experiments in each irradiation protocol (rather than mean and standard deviations) was plotted against the irradiation dose. To exclude the initial shoulder at very low doses (Appendix I) from consideration the line of best fit for the linear

portion of the curve was determined as that having the highest correlation coefficient when all of the quadruplicate values were included in the computations. With the single exception of cells irradiated immediately before PHA stimulation (section 7.2.3.2; scheme B of Fig. 7.A) the straight line curve was fitted through all points corresponding to all irradiation doses.

The calculations of D_0 and extrapolation numbers (n) are explained in Appendix I. In brief D_0 represents the irradiation dose causing the surviving fraction to be reduced to 37% whereas the extrapolation number is determined as the Y-axis intercept when the linear portion of the survival curve is extrapolated back to zero dose and represents an ability to build up and/or repair sublethal irradiation damage.

7.3 RESULTS

7.3.1 Post-irradiation cell recoveries and viabilities

Tables 7.1 and 7.2 show that the percentage of cells recovered after the preincubation cultures was constant for all irradiation doses with both the usual 20 h preincubation cultures and the extended 44 h cultures. The viabilities of these recovered cells were also unaffected by irradiation dose and length of culture (Tables 7.3 & 7.4).

7.3.2 Post-irradiation E rosette capacities

It can be seen from Tables 7.5 and 7.6 that radiation had no effect on the percentage of cells forming E rosettes after both the 20 h and 44 h cultures.

Table 7.1

Effect of irradiation on the percentage of cells recovered from the 20 h preincubation culture

Dose (Gy)	Experiment number				$\bar{X} \pm \text{SEM}$
	I	II	III	IV	
0	75	52	39	39	51.2 ± 8.5
1	50	45	44	39	44.5 ± 2.3
2	75	41	44	39	49.8 ± 8.5
2.5	60	49	56	56	55.3 ± 2.3
3	83	60	28	58	57.3 ± 11.3
4	75	52	33	50	52.5 ± 8.6

Cells were irradiated and incubated for 20 h in the presence of PHA according to scheme B of Fig 7.A. Results represent the number of cells recovered as a percentage of the total number of cells cultured.

Table 7.2

Effect of irradiation on the percentage of cells recovered from the 44 h preincubation culture

Dose (Gy)	Experiment number				$\bar{X} \pm \text{SEM}$
	I	II	III	IV	
0	50	45	50	57	50.5 ± 2.5
1	45	44	40	57	46.5 ± 3.7
2	33	47	40	61	45.3 ± 6.0
3	45	35	35	50	41.3 ± 3.8
4	53	38	40	50	45.3 ± 3.7

Cells were irradiated and then incubated for 24 h. PHA was added following this culture and the cells were incubated for a further 20 h according to scheme A of Fig 7.A. Results represent the number of cells recovered as a percentage of the total number of cells cultured.

Table 7.3

Effect of irradiation on the percentage viability of the cells recovered from the 20 h preincubation

Dose (Gy)	Experiment number				$\bar{X} \pm \text{SEM}$
	I	II	III	IV	
0	89	81	83	72	81.3 ± 3.5
1	84	82	87	66	79.8 ± 4.7
2	89	83	81	81	83.5 ± 1.9
2.5	85	86	76	94	85.3 ± 3.7
3	88	83	75	90	84.0 ± 3.3
4	81	87	78	85	82.8 ± 2.0

Cells were irradiated and incubated for 20 h in the presence of PHA according to scheme B of Fig 7.A. Results represent the percentage viability of the total number of cells recovered.

Table 7.4

Effect of irradiation on the percentage viability of the cells recovered from the 44 h preincubation

Dose (Gy)	Experiment number				$\bar{X} \pm \text{SEM}$
	I	II	III	IV	
0	91	93	71	81	84.0 \pm 5.1
1	90	92	74	84	85.0 \pm 4.0
2	87	90	69	75	80.3 \pm 5.0
3	84	81	73	86	81.0 \pm 2.9
4	82	78	63	84	76.8 \pm 4.8

Cells were irradiated and then incubated for 24 h. PHA was added following this culture and the cells were incubated for a further 20 h according to scheme A of Fig 7A. Results represent the percentage viability of the total number of cells recovered.

Table 7.5

Effect of irradiation on E rosette forming cells recovered from the 20 h preincubation culture

Dose (Gy)	Experiment number				$\bar{X} \pm \text{SEM}$
	I	II	III	IV	
0	60	58	64	72	63.5 ± 3.1
1	56	62	67	70	63.8 ± 3.1
2	58	64	65	48	58.8 ± 3.9
2.5	70	64	68	43	61.3 ± 6.2
3	65	67	73	45	62.5 ± 6.1
4	72	72	60	58	65.5 ± 3.8

Cells were irradiated and incubated for 20 h in the presence of PHA according to scheme B of Fig 7.A. Results represent the number of E rosette forming cells as a percentage of the total number of cells recovered.

Table 7.6

Effect of irradiation on E rosette forming cells recovered from the 44 h preincubation culture

Dose (Gy)	Experiment number				$\bar{X} \pm \text{SEM}$
	I	II	III	IV	
0	60	58	61	65	61.0 \pm 1.5
1	60	67	60	67	63.5 \pm 2.0
2	61	66	59	63	62.3 \pm 1.5
3	66	58	62	70	64.0 \pm 2.6
4	63	64	71	72	67.5 \pm 2.3

Cells were irradiated and then incubated for 24 h. PHA was added following this culture and the cells were incubated for a further 20 h according to scheme A of Fig 7.A. Results represent the number of E rosette forming cells as a percentage of the total number of cells recovered.

7.3.3 Colony and cluster formation

7.3.3.1 Irradiation of resting lymphocytes:

a) 24 h before exposure to PHA

When resting lymphocytes were irradiated 24 h before exposure to PHA there was a dramatic reduction in colony production (Fig 7.1A). The corresponding survival curve expressed an initial shoulder followed by a linear or exponential region (Fig 7.2A). The width of the shoulder was reflected by the extrapolation value of 2.3 while the slope of the linear region was reflected in the D_0 value of 1.0 Gy. The curve, which was fitted through all the points corresponding to 1.0 to 4.0 Gy, closely approximated a straight line with a correlation coefficient of -0.97.

The reduction in cluster numbers was similar to the reduction in colony numbers. Fig 7.3A demonstrates that the decrease in clusters with increasing radiation dose was slightly more gradual than that of colonies.

7.3.3.2 Irradiation of resting lymphocytes:

b) immediately before exposure to PHA

Lymphocytes irradiated immediately prior to PHA stimulation were greatly inhibited in their colony forming ability (Fig 7.1B). The survival curve for colony growth had both a shoulder and a linear (exponential) region (Fig 7.2B). The extrapolation number for this curve was 5.7, D_0 was 0.8 Gy and the correlation coefficient of -0.95 demonstrated that the linear region was a close approximation to a straight line.

As shown in Fig 7.3B the reduction in colonies was followed by a similar but more gradual reduction in clusters.

7.3.3.3 Irradiation of PHA-treated lymphocytes:

c) 20 h after PHA exposure

In preliminary experiments no difference was found when PHA-treated cells were irradiated as either liquid suspensions in tissue culture tubes or as semisolid agar suspensions in tissue culture plates (Table 7.7). All future irradiations were performed with the cells suspended as agar cultures.

Irradiation of PHA-treated lymphocytes dramatically reduced colony numbers with doses as low as 0.5 Gy demonstrating an effect (Fig 7.1C). The straight line of the survival curve, which was fitted through all of the plotted values, had a correlation coefficient of -0.97, while the value for D_0 was 0.8 and the extrapolation number of 1.2 reflected the absence of a shoulder region (Fig 7.2C).

As shown in Fig 7.3C the reduction in colonies was followed by a similar but more gradual reduction in clusters.

7.3.3.4 Irradiation of PHA-treated lymphocytes:

d) 3 days after exposure to PHA

Irradiation of lymphocytes 3 days after exposure to PHA inhibited colony production with doses as low as 0.5 Gy demonstrating an effect (Fig 7.1D). The survival curve shown in Fig 7.2D did not have a shoulder region and this was reflected by the extrapolation number of 1.1. The D_0 value was 1.0 Gy and the correlation coefficient for the curve, which was plotted through all the values, was -0.93.

Unlike the previous sections the decrease in the number of clusters (Fig 7.3D) did not directly follow the reduction in colony formation. The development of clusters appeared to be more resistant than that of the colonies or clusters from the previous sections.

7.3.4 Direct comparison of survival curves.

A direct comparison of the survival curves for colony formation is demonstrated in Fig 7.2. It can be seen that although the slopes of the curves showed only slight variations, the widths of the shoulder regions varied significantly. The largest shoulder was produced when cells were irradiated immediately before PHA exposure. A smaller but significant shoulder resulted when cells were irradiated 24 h before PHA exposure. No shoulders were demonstrated when the cells were irradiated 20 h or longer after PHA exposure. These results can be re-expressed as the survival curve parameters of extrapolation number (n) and D_0 . As shown in Table 7.8 the values for D_0 were similar with each of the irradiation protocols. The extrapolation numbers differed considerably with different irradiation protocols. The highest value ($n = 5.7$) was produced when the cells were irradiated immediately prior to PHA exposure (scheme B of Fig 7.A) and the next highest value ($n = 2.8$) was obtained when cells were irradiated 24 h prior to PHA exposure (scheme A of Fig 7.A). When cells were irradiated either 20 h or 3 days after PHA exposure (schemes C & D of Fig 7.A) the extrapolation numbers were further reduced to 1.2 and 1.1 respectively.

7.3.5 Variation between individual cultures.

The absolute colony numbers from all experiments are given in Appendix II. A comparison of the curves for a single irradiation protocol revealed that although the absolute colony counts varied, similar trends for colony growth and irradiation dose were produced. When the colony counts were re-expressed as surviving fractions and plotted on survival curves as individual points the reduction in the number of colonies was similar for each irradiation dose (Fig 7.2).

Fig 7.1

Dose response curves for T colony formation when the cells were irradiated at different stages of activation.

(A) Irradiation 24 h prior to PHA exposure; scheme A, Fig 7.A.

(B) Irradiation immediately prior to PHA exposure; scheme B, Fig 7.A.

(C) Irradiation 20 h after PHA exposure; scheme C, Fig 7.A.

(D) Irradiation 3 days after PHA exposure; scheme D, Fig 7.A.

Four curves are shown representing all of the irradiation protocols.

Results represent the mean and standard deviation about the mean of triplicate cultures. (See appendix II for the dose response curves from all of the quadruplicate experiments.)

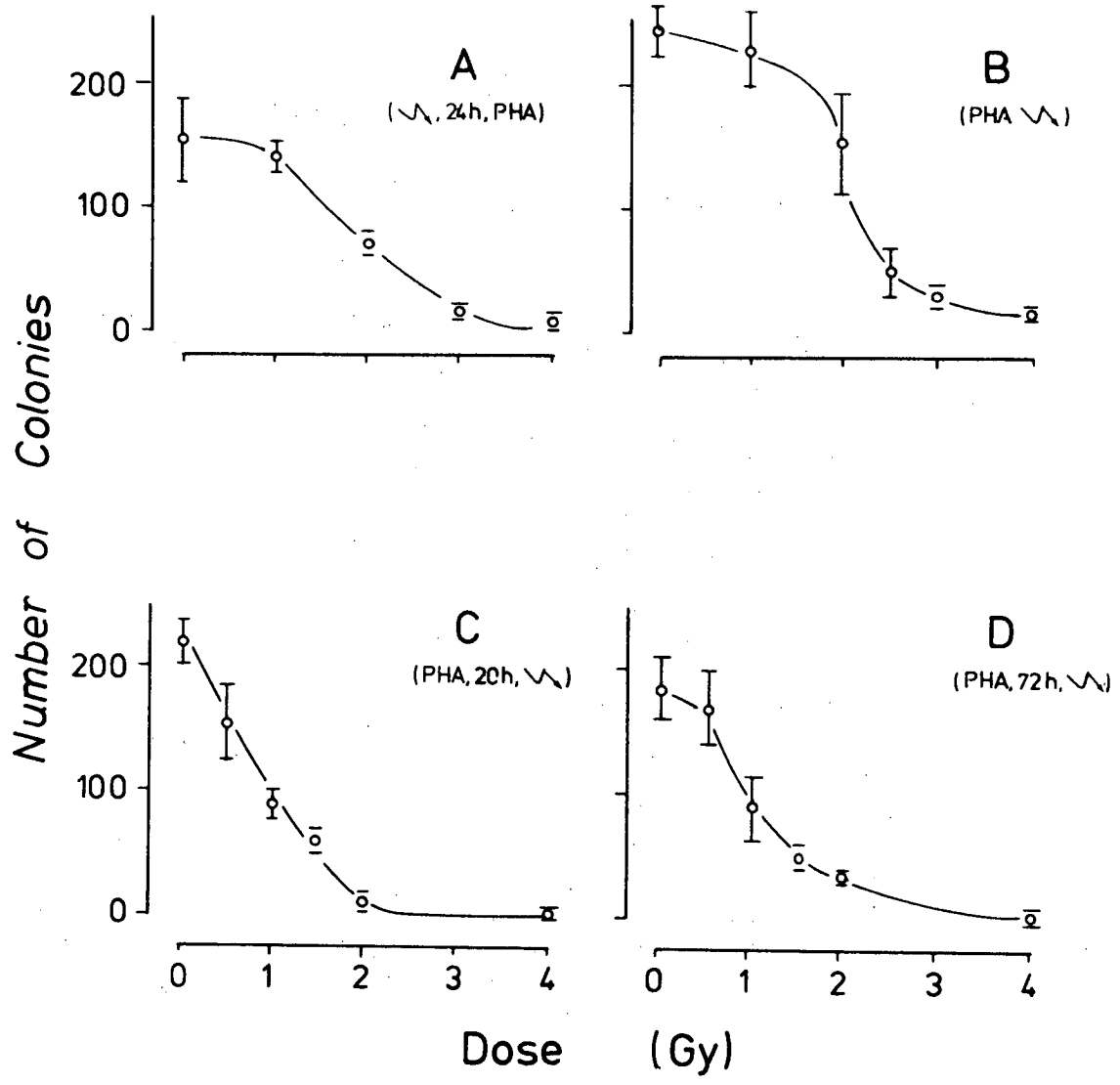


Fig 7.2

Survival curves for T colony formation when the cells were irradiated at different stages of activation.

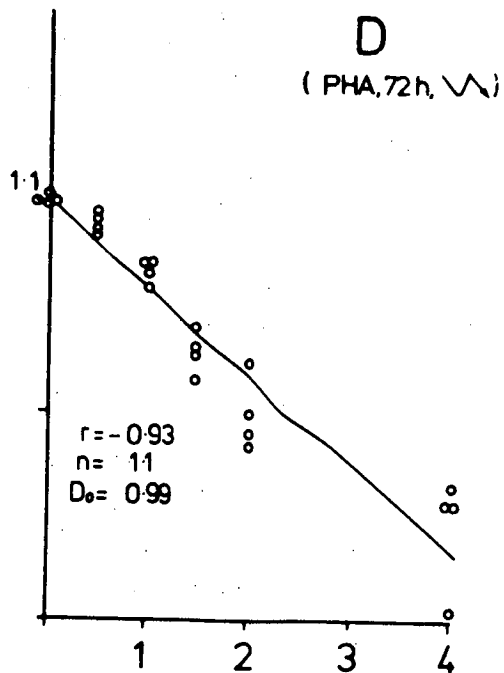
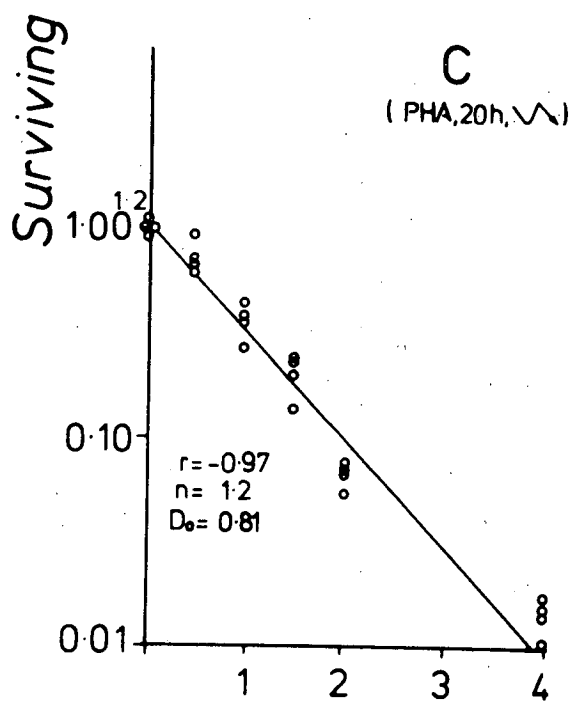
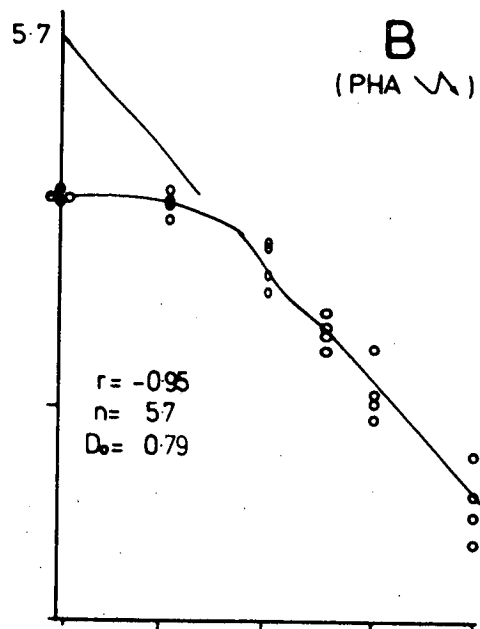
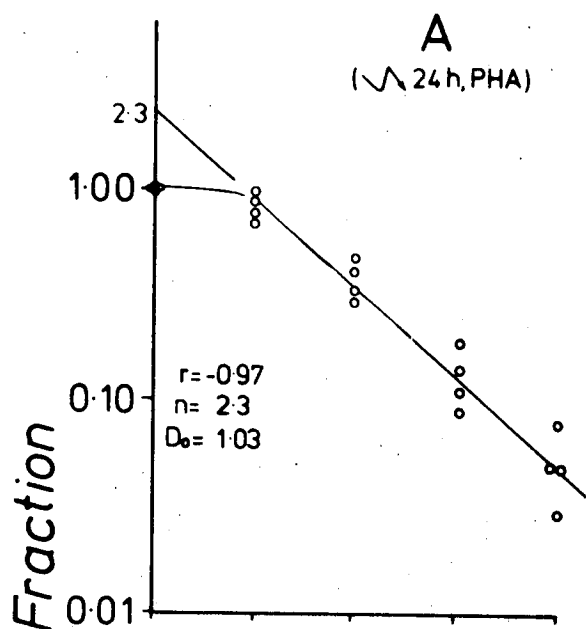
(A) Irradiation 24 h prior to PHA exposure; scheme A, Fig 7.A.

(B) Irradiation immediately prior to PHA exposure; scheme B, Fig 7.A.

(C) Irradiation 20 h after PHA exposure; scheme C, Fig 7.A.

(D) Irradiation 3 days after PHA exposure; scheme D, Fig 7.A.

Each point represents the surviving fraction. The results of four experiments are shown.



Dose (Gy)

Fig 7.3

Survival curves for cluster formation when the cells were irradiated at different stages of activation.

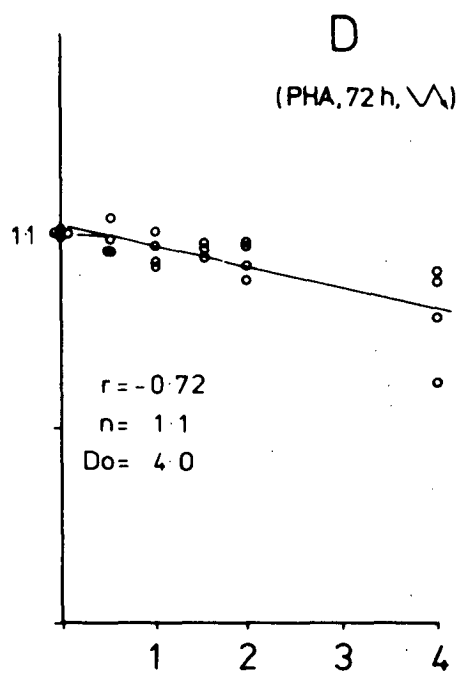
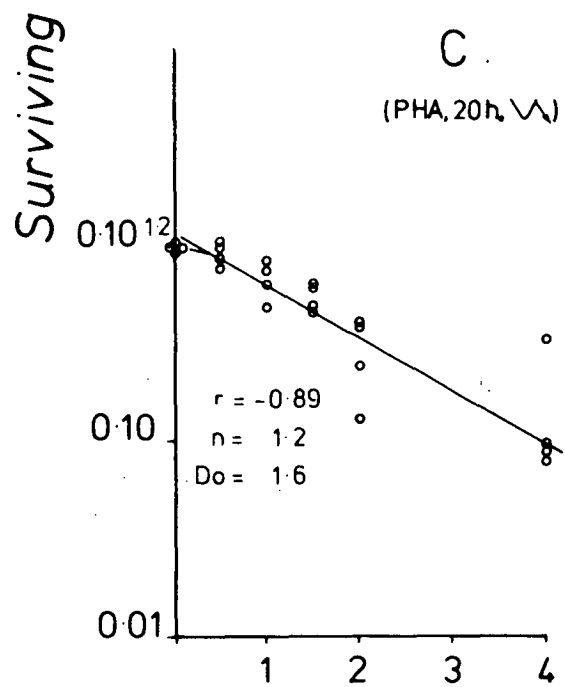
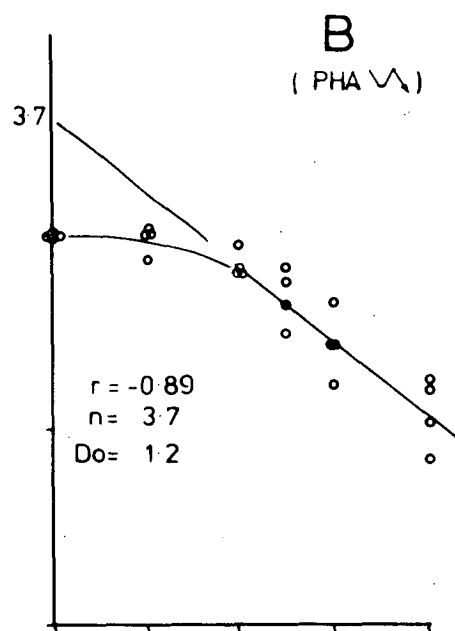
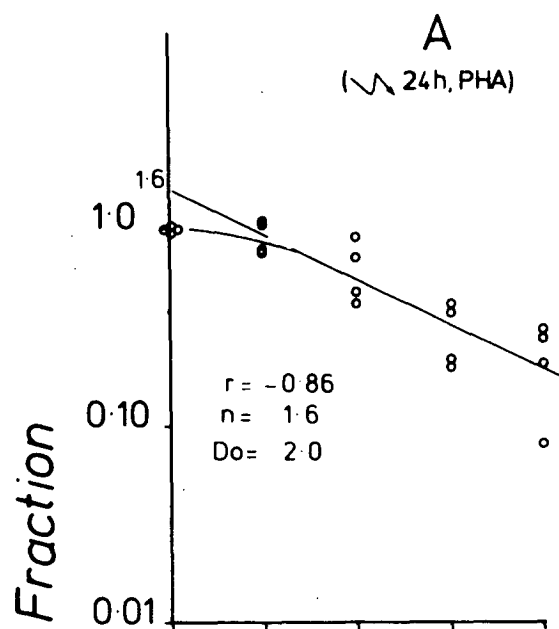
(A) Irradiation 24 h prior to PHA exposure; scheme A, Fig 7.A.

(B) Irradiation immediately prior to PHA exposure; scheme B, Fig 7.A.

(C) Irradiation 20 h after PHA exposure; scheme C, Fig 7.A.

(D) Irradiation 3 days after PHA exposure; scheme D, Fig 7.A.

Each point represents the surviving fraction obtained when clusters (10-49 cells) were scored instead of colonies (>50 cells). The results of four experiments are shown.



Dose (Gy)

Table 7.7

Comparison of the effect of irradiation on PHA-treated cells when irradiated as liquid suspensions or as agar suspensions

Dose	Irradiation performed	
	liquid phase	agar phase
0	610 \pm 65	570 \pm 85
1	275 \pm 60	310 \pm 43
2	68 \pm 19	46 \pm 9

Results represent the mean \pm the standard deviation about the mean when 50 000 PHA-treated cells were irradiated in polystyrene tubes (liquid phase) or culture dishes (agar phase). A representative experiment is shown.

Table 7.8

D_0 values and extrapolation numbers (n) for cells irradiated at different stages of colony formation

IRRADIATION SCHEME	D_0	n
RESTING CELLS:- (24 h before PHA exposure) (scheme A Fig 7.A)	0.98	2.3
----- RESTING CELLS:- (immediately before PHA) (scheme B Fig 7.A)	0.79	5.7
----- STIMULATED CELLS:- (20 h after PHA exposure) (scheme C Fig 7.A)	0.81	1.2
----- STIMULATED CELLS:- (3 days after PHA exposure) (scheme D Fig 7.A)	0.99	1.1

Lymphocytes exposed to radiation had a greatly reduced ability to divide and form colonies. This finding is consistent with the well established view that lymphocytes represent one of the most radiosensitive of all mammalian cells (Stewart and Perez, 1976). Analysis of the survival curves provided evidence that lymphocytes, at early stages of activation, were able to withstand higher irradiation doses before a loss of mitotic ability was registered.

As summarised in Table 7.8 and Fig 7.2 the main difference between the survival curves for all of the irradiation protocols was the extrapolation number (or the shoulder region of the curves) i.e. the cell's ability to withstand low irradiation doses without suffering a loss of viability or replicative capacity. Although there was heterogeneity with these extrapolation numbers the D_0 values (or the cell's overall sensitivity) did not show significant variation suggesting that the single difference for colony forming cells and their radiation sensitivity at different stages of activation was related to their ability to withstand higher irradiation doses.

D_0 values for most, but not all, lymphocyte populations have been calculated to be around the range of 0.75-1.0 Gy (Stewart and Perez, 1976) and the survival curves, determined from non-colony forming techniques, were usually exponential (Sprent, Anderson and Miller, 1974; Stewart and Perez, 1976; Baral and Blomgren, 1976). The D_0 values shown in Table 7.8 were within this (0.75 - 1.0 Gy) range but the survival curves were either exponential or sigmoidal (Fig 7.2) depending on the timing of irradiation and PHA exposure. As sigmoid or shouldered curves are characteristically produced by cells that

withstand sublethal damage (Appendix I) it would appear that the timing of irradiation in relation to PHA exposure was an important factor for this phenomenon to be demonstrated with lymphocyte colony formation.

Schrek and Stefani (1964) found a protection from the cytotoxic effects of irradiation (as determined by phase contrast microscopic analysis) when PHA was added to lymphocyte suspensions. Similar protective effects have been reported by other workers using a variety of systems which effectively measure interphase death or death of non-dividing cells (Schrek and Stefani, 1964; Cirkovic, 1969; Conard, 1969; Sato and Sakka, 1970; Sprent et al., 1974; Anderson and Warner, 1976). One may speculate that PHA or antigen stimulation can protect cells from the cytotoxic effects of irradiation due to an increased metabolic activity. This radioprotection from interphase death does not necessarily extend to protection from mitotic death as PHA stimulated lymphocytes, which have survived doses of irradiation that destroyed non-stimulated cells, demonstrated a reduced capacity to proliferate (Conard, 1969; Rickinson and Ilbery, 1971; Anderson and Warner, 1976) and mitotic death resulted due to chromosomal damage (Rickinson and Ilbery, 1971). This was supported by Baral and Blomgren (1978) and Onsrud and Thorsby (1980) who, using liquid culture cell division assays, presented evidence demonstrating that there was no difference in the radiosensitivity of mitogen or antigen primed and unprimed lymphocytes provided that certain culture conditions were applied. It is possible that stimulation protects lymphocytes from interphase death through a system of repair mechanisms which are efficient in conserving the integrity of the cell but unable to repair the chromosomal aberrations that become critical during cell division.

Most evidence suggests that mitotic death cannot be avoided whereas interphase death may be protected against or even repaired (Anderson and Warner, 1976). Despite this general view there are occasional reports suggesting that PHA and other mitogens can protect lymphocyte division from irradiation damage. Ulmer and Flad (1981) demonstrated that irradiated mononuclear cells formed colonies if they were incubated with PHA in liquid culture prior to seeding in agar. If PHA was omitted from this culture there was no colony growth. Circovic (1969) found that DNA and RNA synthesis was protected by PHA pretreatment while Vaughan-Smith and Ling (1974) found a decrease in lymphocyte radiosensitivity, as measured by tritiated thymidine uptake, when lymphocytes were treated with Con A immediately prior to irradiation. They also found a decrease in lymphocyte stimulation when the cells were irradiated and exposed to Con A at increasing time intervals later. These results suggest that the timing of stimulation and irradiation is critical for a radioprotective effect to be demonstrated. It must however be remembered that, as already discussed, blast transformation may not necessarily reflect a cell's mitotic capability because irradiation has been shown to stimulate the uptake of tritiated thymidine (Evans and Norman, 1968).

It is reasonable to assume that the genome is the primary target for radiation-induced damage when the effect is registered as mitotic death. For mitotic death to be prevented it would be necessary for "nicks" in DNA molecules or chromosomes to be repaired. Evidence for such a possibility has been obtained from numerous sources. Normal human lymphocytes, in the absence of any ligands, are stimulated to incorporate tritiated thymidine into their DNA following exposure to radiation (Painter and Cleaver, 1967; Evans and Norman, 1968). It has

been proposed that this DNA synthesis is due to a form of repair (Cleaver, 1969). These cells, in the presence of fresh medium, respond to PHA by undergoing blast transformation and cell division (Evans and Norman, 1968) while lymphoma cells grown in tissue culture can efficiently repair radiation induced breaks of DNA strands (Lett et al., 1967; Ormerod and Stevens, 1971). On this basis it is possible that lymphocytes possess the ability to repair damaged DNA through the activities of repair enzymes. Resting lymphocytes are deficient in the enzymes DNA polymerase and DNA ligase (Loeb, Agarwal and Woodside, 1968; Pedrini et al., 1971) and as these enzymes appear to be essential for DNA repair processes (Regan et al., 1971; Town et al., 1971) their deficit might be an explanation for the inability of resting lymphocytes to repair radiation damaged DNA. If lymphocytes are stimulated by PHA the activities of DNA polymerase and ligase are dramatically elevated (Loeb et al., 1968; Pedrini et al., 1971) suggesting that PHA stimulation enabled the functional expression of these "repair enzymes". As PHA stimulation can protect cells from interphase death it is likely that the elevated activity of these "repair enzymes" corresponded to the increased radioresistance; strong evidence that PHA activated lymphocytes possess the necessary framework to repair radiation-damaged DNA strands. In addition intact chromosome repair mechanisms have been demonstrated in stimulated lymphocytes (Prempre and Merz, 1969). This finding is supported with established cell lines (Drewinko and Humphrey, 1971) and evidence has been documented that the activation of resting lymphocytes is also accompanied by a development of a chromosome break-repair apparatus (Prempre and Merz, 1969; Stefanescu et al., 1972). In addition (blast) transformed lymphocytes can repair single-stranded breaks of

DNA at 10 times the rate of non-transformed lymphocytes (Hashimoto, Ono and Okada, 1975). Thus it appears likely that resting cells are unable to repair single-stranded DNA excisions due to a deficiency in enzyme activity but during the process of activation the repair mechanisms become functional (Prempre and Merz, 1969; Stefanescu et al., 1972).

The major differences between the survival curves of Fig 7.2 are the widths of the non-linear or shoulder regions. With the evidence supporting a functional presence of repair enzymes in activated lymphocytes the differences in these curves can be interpreted in terms of the presence of repair enzymes. Lymphocytes irradiated immediately prior to PHA exposure (Fig 7.2B) produced a survival curve with a significant shoulder ($n = 5.6$); indicative of an ability to repair radiation-induced damage and, as PHA was added immediately after irradiation, it is likely that the responsible repair mechanisms were also activated immediately. Support for this comes from the survival curve produced when lymphocytes were irradiated 24 h before exposure to PHA (Fig 7.2A) as a shoulder ($n = 2.3$) was produced suggesting operative repair mechanisms. As irradiation effects of mitosis may take 2-3 days to be expressed (Rickinson and Ilbery, 1971) it is plausible that some of this damage was repaired before the effect became irreversible. At later stages of differentiation or activation the mitotic repair mechanisms were ineffective. This can be derived from the relevant survival curves because they did not contain shoulders and the extrapolation numbers approached unity (Figs 7.2C & 7.2D). It therefore appears likely that damage to a cell's mitotic apparatus can be restored through the activities of repair enzymes or mechanisms which are more effective

during early rather than late stages of activation. Alternatively these "repair enzymes" might be more effective when in a liquid culture rather than an agar culture phase. It is possible that the direct cell:cell contact favoured by the liquid culture might promote the repair enzyme activity associated with PHA-exposure. The results in Table 7.7 showing that PHA-treated cells irradiated as either liquid or agar suspensions were equally sensitive to irradiation, to some extent argue against this proposition. Furthermore, early liquid culture experiments looking at blast transformation failed to demonstrate a radioprotective effect (Rickinson and Ilbery, 1971; Herva and Kiviniitty, 1975; Baral and Blomgren, 1976). However, to investigate this possibility in the present system 20 h PHA-treated cells should be irradiated and then incubated for a further 20 h in liquid phase before being seeded into agar; this was not performed with the present work. Nevertheless the results reported herein do in fact demonstrate that lymphocytes, as reflected by a shouldered survival curve, can withstand higher doses of irradiation during their early activation stages (albeit whilst in a liquid culture phase). Such a finding had not previously been reported.

The expression of the "repair enzymes" at an early stage of activation may explain a number of the contradictory reports concerning lymphocyte cell division and the possible protective effects produced from exposure to PHA. The timing of irradiation and PHA stimulation seems crucial for repair enzyme activity to correspond to a period of radiation damage. Protection from radiation-induced damage was most effective if the cells were irradiated at the early stages of activation, i.e. when the repair enzymes are the most active. It is possible that the enzymes responsible for repairing

damaged DNA molecules are concomitantly activated during the increased metabolic activity characteristic of lymphocyte activation. Although the activity of these enzymes decreased at later stages this does not preclude other enzymes, that may repair cytoplasmic damage, from functioning as interphase death can be prevented at various stages of activation (Schrek and Stefani, 1964).

In the idealised situation colonies will appear after a cell completes six mitotic cycles to produce more than 50 daughter cells ($2^6 = 64$). If, as has been reported, irradiation either significantly delays the onset of the first mitotic cycle (Rickinson and Ilbery, 1971) or extends the length of each individual cycle to such an extent that a cell is unable to complete these six mitotic cycles, a cluster will develop instead of a colony. Thus if the major effect of irradiation was to reduce the total number of cell divisions by delaying and/or extending each mitotic cycle an apparent radioresistance of cluster forming cells would be observed because the colony forming cells would give rise to clusters instead of colonies. In the experiments described in Figs 7.2 & 7.3 both colony and cluster formation was reduced simultaneously suggesting that this was not the cause and therefore irradiation produced mitotic death for both colony and cluster forming cells. The only exception was when cells were irradiated 3 days after PHA exposure (Figs 7.2D & 7.3D). The appearance of clusters seemed to be relatively radioresistant as a dose of 4 Gy reduced the number of colonies to less than 4% of the original population whereas clusters were only reduced to 40%. At this stage of irradiation the stimulated T lymphocyte colony forming cells were beginning to undergo cell division (section 3). The appearance of clusters might be due to a delay in mitotic death (Anderson and

Warner, 1976) which would allow limited cell division and therefore cluster formation. In addition some clusters would have formed by the time the cultures were irradiated. With this reservation it can be assumed that cells giving rise to either colonies or clusters are equally sensitive to mitotic death and as such are likely to be derived from the same population of cells.

As discussed in sections 5 & 6 T lymphocyte colony formation requires cellular interactions between colony forming cells and helper cells. As helper cell defects would prevent colony production the radiosensitivity of these helper cells must therefore be considered in an evaluation of the effects of irradiation on T colony growth. From independent studies it is highly improbable that the irradiation-induced reduction in T colony growth was due to an inhibition of helper cell activity as helper T cell functions are radioresistant (Anderson and Warner, 1976; Fauci et al., 1978). In addition, purified lymphocytes stimulated by PHA can be induced to form colonies in the presence of leucocytes irradiated with 2 Gy (Foa and Catovsky, 1979; Lowenberg and de Zeeuw, 1979). These irradiated leucocytes presumably contain helper T cells and monocytes that do not proliferate but provide essential helper activity for the proliferating cells. The release of the important growth factors is resistant to the doses of radiation that prevent colony formation. The inhibition of colony growth is therefore due to a direct effect on the colony forming cells rather than an indirect effect via helper cells and/or their activities.

As the technique for growing T lymphocyte colonies required a liquid preincubation followed by an agar culture it was necessary to ascertain that the cells seeded into the agar medium were

representative of the original population incubated during the liquid culture. If irradiation produced a reduction in the number of cells recovered after the preincubation cultures then the cells seeded into the agar medium may not represent the original population as radioresistant cells would survive the irradiation and the preincubation cultures; in contrast the radiosensitive cells would be eliminated. Such an occurrence will increase the proportion of radioresistant cells seeded into the agar and therefore an unrealistically high proportion of radioresistant cells would be assayed for colony growth. The results in Tables 7.1 to 7.6 do not show a reduction in either the total number of cells recovered, their viability or their E-rosette capacity for both the 20 h and 44 h cultures.

The sensitivity of T lymphocyte colony growth was consistent with the findings of Gelfand et al. (1981) who found that 2 Gy was sufficient to produce a 50% reduction in colony formation. The general assumption that colony formation is a radiosensitive event might be made were it not for the results of Ulmer and Flad, (1981) who claim that doses as high as 10 Gy were not inhibitory. The differences between these results might be due to the nature, or origin, of the cells responsible for the generation of T colonies. Within the total population of lymphocytes radiosensitive and some relatively radioresistant fractions exist (Anderson and Warner, 1976; Baral and Blomgren, 1976; Gupta and Good, 1977; Kwan and Norman, 1977; Anderson and Lefkovits, 1980; Birkeland, 1980) while within the subpopulation of PHA-responsive T lymphocytes at least two further subpopulations may be defined in respect to their responsiveness to Con A (Gelfand et al., 1981) and their radiosensitivities (Anderson

and Warner, 1976; Baral and Blomgren, 1976). Furthermore, cell fractionation studies have revealed that T lymphocyte colony precursor cells are contained within a subpopulation of PHA reactive lymphocytes (Swart and Lowenberg, 1978). Cells responsive to PHA therefore consist of subpopulations that differ with respect to both colony forming ability and radiation sensitivity. It is therefore likely that the radioresistance of colony formation seen in a particular study will depend on the exact culture conditions used since different subpopulations of lymphocytes, varying in radiosensitivity, might be selectively favoured. Knox, Shifrine and Rosenblatt (1982) examined the proliferative capacity of irradiated T lymphocytes cultured from whole (unseparated) blood and found that the number of colonies relative to dose followed a bi-exponential curve suggesting that two distinct populations of lymphocytes (radiation sensitive and relatively radiation resistant) are capable of colony formation.

The method of Ulmer and Flad utilized microcapillary tubes and agar at a final concentration of 0.2% whereas the method described in this thesis required the higher agar concentration of 0.5% and the colonies were grown in petri dishes. It is likely that the essential differences between the colonies are due to this final agar concentration. The colonies produced with 0.2% agar consisted of tightly compacted aggregates of cells (Ulmer and Flad, 1981) suggesting a lack of motility of these cells compared to the colonies grown in 0.5% agar which were more disperse and therefore more motile. The basic differences in these colony forms on both morphological appearance and radiation sensitivity is evidence that T lymphocyte colonies produced by the two systems may select for cells from different subpopulations of T lymphocytes.

At low irradiation doses lymphocytes exhibited a limited capacity to divide as shown by their reduced ability to form colonies. This extreme radiosensitivity was consistent with established findings obtained from alternative culture systems, however, the radiosensitivity of colony formation varied according to the timing of irradiation in relation to the stage of colony growth. Through survival curve analysis it was shown that the colony forming cells could withstand higher irradiation doses without losing their replicative ability when they were irradiated during early stages of PHA-stimulation. A similar "protection" by PHA-stimulation has been well documented for non-dividing lymphocyte functions but, due to the lack of a suitable methodology, had hitherto not been described for dividing lymphocytes. By analogy with liquid culture systems this protective effect could be explained in terms of "repair enzymes". The activity of these enzymes was manifested during the early stages of activation whilst the cells were in a liquid culture phase. It is plausible that they were concomitantly activated with the increased metabolic activity characteristic of the early activation events after PHA exposure. At later stages of activation (during the agar culture) this ability to repair radiation-induced damage was diminished as reflected by the corresponding extrapolation numbers (n) which approached unity. The decreased colony formation was not due to a curtailing in the number of cell cycles and was unlikely to be due to accessory cell defects. These results provide evidence that PHA-stimulation can, to a limited degree, prevent mitotic death of lymphocytes provided the cells are irradiated at early activation stages.

SECTION EIGHT

CONCLUDING DISCUSSION

8.1 Introduction

The studies throughout this thesis examined the growth of human T lymphocytes in semisolid medium. The technique was described in section 2 and further analysed in section 3. The results in section 4 examined the peculiar requirement for SRBC while sections 5 & 6 investigated in detail the cellular interactions involved in colony formation. The T lymphocyte colony assay was utilised in section 7 to determine the effect irradiation had on proliferating T lymphocytes. In this section some general aspects of the colonies are discussed in relation to the results obtained by other investigators. In addition the advantages of T lymphocyte colony assays over other methods of studying T lymphocytes are considered as well as their future applications.

8.2 Colony growth

The majority of colonies discussed throughout this thesis developed within the agar matrix and varied in size from small (50-100 cells) to large (>500 cells); clusters (10-49 cells) were also present. Fibach et al. (1976) originally defined colonies developing within the agar as type I and those growing on the surface of the agar as type II. According to this criterion the colonies described herein were usually of the type I category. Under different culture conditions other workers have found the type II category to be more common (Fibach et al., 1976; Zeevi, Goldman and Rozenszajn, 1977; Rozenszajn et al., 1981). It has been suggested that these colonies (type I and II) developed from different functional populations of T lymphocytes (Rozenszajn et al., 1981). An examination of the literature provides an interesting observation; colonies of type II characteristics were the common-type when either low agar

concentrations ($<0.4\%$), methyl cellulose or liquid overlayers were used, whereas type I-like colonies were the common-type when higher (0.4% or above) agar concentrations were used (Table 1.1). The concentration of agar is therefore the critical factor for type I or type II colonies. As discussed in section 2 the high agar concentration restricted the migration of the highly mobile colony cells. The formation of type I and type II colonies may therefore relate to the extent of migration of these cells. At low agar concentrations the colony cells can migrate to the surface of the agar where they aggregate to produce flattened type II colonies, at higher agar concentrations this excessive movement is restricted and type I colonies develop within the agar. It appears that type II colonies emerge from the combination of migration, aggregation and proliferation whereas type I colonies develop through proliferation only. Rozenszajn et al. (1981), by harvesting and subculturing type I and type II colonies, found that only type II colonies were produced whereas Smith and Sachs (1979) demonstrated both type I and type II colonies when type II colonies were subcultured. These results support the possibility that the different colony types result as a consequence of the extent of their migration rather than originating from different progenitor cells. If a major difference existed, type II colonies would always produce type II colonies and type I would always produce type I colonies. It is unlikely that cells developing into T lymphocyte colonies belong to a unique subpopulation of lymphocytes, even though only 1% (approximately) form colonies. Claesson et al. (1981) showed that colony cells comprised a mixture of Leu 3a and Leu 2a phenotypes, although Leu 3a predominated. This heterogeneity of colony cells has also been reported by Triebel et al.

(1981) and Fierro et al. (1982). In addition Bockman and Rothschild (1979) demonstrated both suppressor and helper activity from T lymphocyte colonies. It therefore appears that colonies of "helper" or "suppressor" phenotype can develop with the helper colonies being more frequent. Further advances concerning the identity of the colony cells require a more specific definition of subpopulations of T lymphocytes. Presently T lymphocytes are defined as either "helper-phenotype" i.e. OKT4+, Leu 3a+ and Fc-IgM+ or "suppressor-phenotype" i.e. OKT8+, Leu 2a+, Fc-IgG+, however there are a number of inconsistencies with these characterisations. With regard to Fc receptors a "switch" from Fc-IgG+ to Fc-IgM+ has been demonstrated (Pichler, Lum and Broder, 1978), indicating that this classification may not be specific. With regard to monoclonal antibody definitions it was originally believed that T lymphocytes were either "helpers" or "suppressors" according to the presence of OKT4 or OKT8 antigens respectively (Reinherz et al., 1979). It is now becoming apparent that these definitions do not sufficiently represent helper and suppressor cells because suppressor activities have been identified in the helper fraction (Thomas et al., 1981) and helper activities (e.g. IL2 production) have been identified in the suppressor fraction (Luger et al., 1982). Although characteristic in vitro activities of helper- and suppressor-phenotype cells are their namesake activities, recently reported work indicates that the major distinguishing factor between these cell types is their ability to respond to different HLA antigens (Lowenthal, personal communication).

Throughout this thesis the analysis of colonies was given priority over that of clusters. For consistency with both the haemopoietic colony systems and the previously published T lymphocyte

colony work the distinguishing factor of 50 cells was used as the lower limit to describe a colony. The value of 50 cells was arbitrary and as shown in Table 1.1 other workers have adjusted this value to accommodate their individual culture systems. The frequency of clusters in the present study was always greater than that of colonies. A similar situation was found with haemopoietic colonies (Metcalf, 1977) and differences between the colony and cluster forming cells of normal individuals are unlikely. Clearly the underlying distinction between clusters and colonies relates to the proliferative capacity of the respective progenitor cells. It is possible that cluster forming cells belong to an "older" population of cells which have a more limited lifespan and mitotic capacity than their "younger" counterparts. Alternatively, as proliferating cell groups range in size from 2 to greater than 500 cells, it is conceivable that clusters simply represent the lower end of this normal range. Further explanations to explain the presence of clusters will include the shortage of, or competition for, growth factors within the culture medium thereby restricting their proliferative capacity, random intracellular events that may produce signals resulting in premature termination of cell growth or, as a result of the migration of colony cells some clusters might be smaller "satellite" colonies that originated from a larger colony. Cells from colonies and clusters were found to have similar densities and sedimentation velocities (Claesson et al., 1977a) while results from section 7 showed that colony and cluster formation was equally sensitive to irradiation. Further, by improving the culture conditions clusters could develop into colonies (section 6) thereby supporting the proposition that cells producing clusters or colonies are likely to be similar.

8.3 Cellular interactions and the value of T lymphocyte colonies

Although cellular proliferation can be assayed with liquid culture systems the semisolid agar technique has the advantage that individual reactions can be studied. The semisolid agar culture system also has the benefit that proliferation of single cells or cell units can be directly observed and responses to regulatory factors can be monitored. There are a number of artefacts to be aware of when tritiated thymidine uptake is used to measure DNA synthesis as a guide to cell division. These include the non-specific labelling of proteins and other substrates by tritiated thymidine, repair of damaged DNA molecules in the absence of cell division, high ($>2 \times 10^6$ /ml) cell concentrations which result in high cell death and blast transformation but limited cell division (Maurer and Laerem, 1976).

The requirement for cellular cooperation was recognised by a number of workers, however, the various technical modifications adopted by these workers (summarised in Table 1.1) effectively removed the dependence of the colony forming cells on both PHA and cellular interactions and therefore the important and integral interactions of colony formation were concealed. The results described in this thesis, in addition to the reported observations of other workers, enabled clarification of the individual reactions of colony formation (section 6).

Although only a small minority of cells (approximately 1%) formed colonies the value of the colony assay lies with the cellular interactions involved rather than the colonies themselves. A colony can therefore be regarded as the "end-product" or index of a number of interdependent reactions. These reactions, fully investigated in

sections 5 & 6, required the involvement, as a minimum, of three cell populations namely the colony forming cells (TLCFC), adherent cells and IL2-producing cells. The TLCFC's were of a mature phenotype as demonstrated by their ability to form E-rosettes and respond to PHA. Adherent cells were essential during the early activation stages only. Although it was predicted that monocytes were responsible for this adherent cell activity it is important to consider the possible involvement of dendritic cells in colony formation in light of the recent developments relating to their role in antigen presentation. Further characterisation and isolation of these cell types is required to fully investigate their role, if any, in colony formation. The remaining category of cells was responsible for the production of IL2. Initially it would have been expected that they would specifically belong to the helper phenotype (OKT4+ or Leu 3a+) class of cells as these cell types were previously shown to release soluble factors with IL2 activity (Reinherz et al., 1980). As recently demonstrated, both helper and suppressor phenotype cells (OKT4+ & OKT8+) can secrete a lymphokine with IL2 activity (Luger et al., 1982). The exact identity of the T cells releasing IL2 therefore remains unclarified. Nevertheless it was possible to propose a model describing the events of colony formation (section 6) and such a model will be beneficial for future studies.

The T lymphocyte colony assay was utilised to determine the effects of irradiation on proliferating T lymphocytes (section 7). Previous studies relating to irradiation and proliferating lymphocytes were restricted to unsatisfactory liquid culture systems. The advantage of the colony system for such an analysis could be attributed to the ability to directly observe colonies of

proliferating cells and to quantitatively analyse the lethal effects of irradiation with conventional radiobiological survival curves.

8.4 Future work

The studies detailed throughout this thesis showed that colony growth was the culmination of a number of interdependent cellular interactions. By applying the model outlined in section 6 it would be possible to analyse in greater detail and with more specificity the steps involved in T cell activation and proliferation. With PHA (or mitogen) as the only stimulus colony production is the end result of a series of reactions including activation of T cells (both colony-forming and IL2-producing), T cell-adherent cell interactions, IL2 production and cellular proliferation. Thus the development of colonies may be regarded as an index of the function of all these cell types and not just the small percentage (approximately 1%) that proliferate to form colonies. Failure of any of these reactions will result in poor colony formation. This system provides a suitable model for studying cellular interactions and the various influences which have important in vivo ramifications. Included in such a category are transplantation rejection phenomena, study of the sites of action of immunomodulatory drugs and the role of suppressor cells in cell mediated immunity.

An interesting point to emerge from the studies involving cytochalasin B was the likely role of the cytoskeletal system of proteins in the processing of antigen and lymphokine. Although only an approximation to the in vivo situation the results suggest that actin might be a critical link in the transmission of messages received by the surface membrane receptors. Further work in this area appears warranted as an understanding of these responses in normal cells may

help elucidate the factors behind lymphocyte malfunction in disease states.

Further applications of the T colony assay relate to the colonies themselves without regard to the cellular interactions required for their formation. With an advance in the preparation and purification of interleukin preparations, colonies of antigen specific T cells could be prepared by substituting PHA with the antigen and incorporating optimal quantities of the interleukins 1 and 2. Other avenues of approach include the expansion of PHA-induced colonies in IL2-containing liquid for functional and surface marker analysis.

A P P E N D I X O N E

RADIOBIOLOGY

I.1 Introduction

Before a discussion of radiobiology can be made it is necessary to define a number of concepts and their derivations. It is the aim of this section to briefly overview some of the more important aspects of cellular radiobiology.

To study the effects of irradiation a measure of cell death is usually performed with the results expressed as survival data and plotted on a survival curve. The concept of cell death in classical radiobiological terms generally refers to reproductive death, i.e. the loss of an unrestricted proliferative ability. The survival curve is a valuable tool for studying proliferative death as it directly relates survival with irradiation dose and can be used to quantitatively compare different cell populations. The axes of a survival curve are the effect (survival) plotted on a logarithmic scale, and the irradiation dose plotted on a linear scale. Survival is defined in terms of surviving fraction i.e. the number of cells surviving the irradiation is expressed as a fraction of the total number of surviving, non-irradiated cells. For example if irradiation caused 40% cell death then the surviving fraction is 0.6 (i.e. 60% survive). From a survival curve it is possible to quantitate other parameters to describe irradiation effects, e.g. extrapolation number and D_0 .

I.2 Irradiation induced damage

Cells killed by irradiation are destroyed by three related processes. 1:- absorption of energy, 2:- production of lesions, 3:- biological expression of these lesions. Absorption of energy occurs when irradiation causes electrons to be set in rapid motion; it is these rapidly moving electrons that produce lesions causing biological

damage (Elkind and Whitmore, 1967). These rapidly moving particles travel along non-defined "tracks" releasing energy through the production of ionizations or excitations, defined as linear energy transfer (LET). X-rays and fast moving electrons produce tracks of low LET i.e. they do not rapidly release energy along their tracks.

I.3 Survival curves

Survival curves can be classified into two types; exponential and sigmoid. Both of these types were demonstrated by Wood (1953) with related haploid and diploid strains of yeast. When the survival data for the haploid strain was plotted on a linear scale an exponential curve resulted (Fig I.1A). By replotting the same data on a semilogarithmic plot the curve became a straight line (Fig I.1B). The survival data from the genetically related diploid strain plotted on a linear scale produced a sigmoidal curve (Fig I.1C). This data replotted on a semilogarithmic plot resulted in a curve that was initially concave downwards but straightened through the remaining points (Fig I.1D). A comparison between the linear and semilog. survival curves shows that differences between the haploid and diploid strains are demonstrated more clearly on the semilog. rather than the linear curves.

I.4 Target theory

The target theory is a concept that has been used by radiobiologists to explain, through both mathematical and physical aspects, the lethal effects of irradiation. Generally, and in physical terms, the concept of target implies that within a cell vital structures exist whose integrity is essential for the cell's survival. These vital "survival" structures are the targets that when

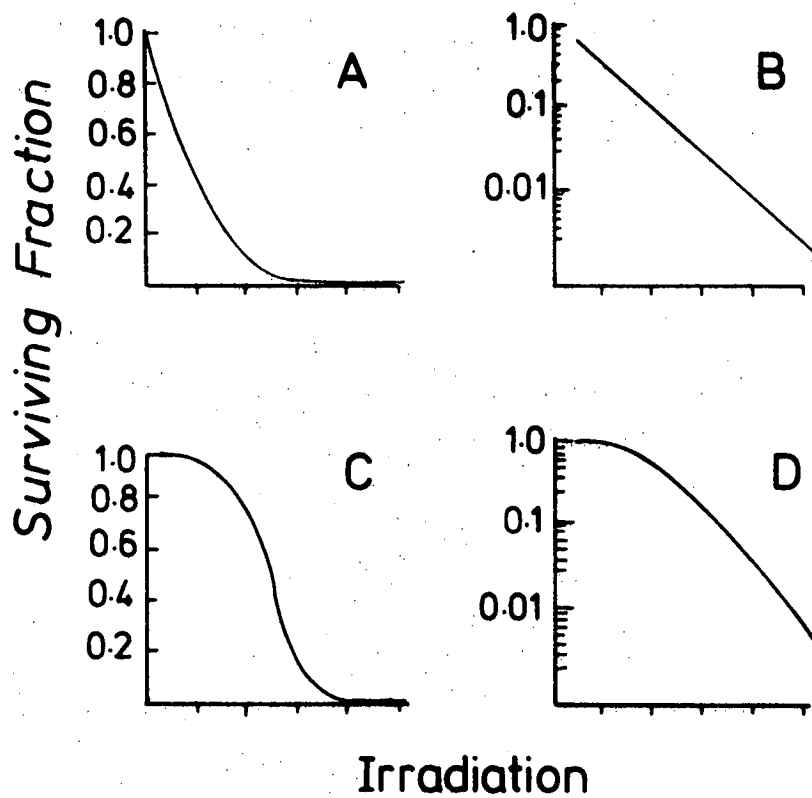


Fig I.1

- A) Exponential survival curve plotted on linear axes
- B) Exponential survival curve plotted on semilogarithmic axes
- C) Sigmoidal survival curve plotted on linear axes
- D) Sigmoidal survival curve plotted on semilogarithmic axes

inactivated or damaged by irradiation cause loss of cell function, as measured by cell death or loss of proliferative capacity. Although the theory of targets has been generally accepted there is no real evidence that targets exist in a form that can be identified or physically isolated. (There is some belief that DNA-strands act as targets.) The target concept is a useful theory which, when used mathematically acts as a tool for calculations and when used in the physical sense aids in the understanding of the underlying mechanisms of radiation induced damage.

I.5 Exponential survival:- Mathematical approach

For cells exposed to low LET radiation each cell will contain a total sensitive volume or target volume of size v . Active events (i.e. radiation induced events producing ionizations) which occur in the target of size v can be regarded as "hits" or "effective events". Active events outside these sensitive targets have no effect and are therefore regarded as misses. The target concept of biological irradiation allows for one or more "hits" (effective events) to occur before a biological effect is expressed, which usually results in cell death. In a cell population the total cell volume exposed to irradiation can be called V while d is the density of active events (active events are directly proportional to irradiation dose). The product of d and V will equal the number of active events (d) scored in a total cell volume (V) and can be designated D i.e. $dV = D$. For an active event to be an effective event (i.e. a hit) it must occur in (or hit) the target (v). The probability that an ACTIVE event is an EFFECTIVE EVENT is determined by the ratio of v/V i.e. for an active event that occurs in the total volume (V) the probability (p) that the

event is an effective event will be determined by the fraction that the target volume is of the total volume. Any active events outside the target are ineffective events or misses.

Equation 1 p (probability of a hit) $= \frac{v}{V}$

The probability that a single cell will be hit h times when D active events occur (i.e. a cell will be hit h times and missed $D - h$ times) can be determined from the equation:-

Equation 2 probability of h hits $= (p^h)(1-p)^{D-h} \left(\frac{D!}{h!(D-h)!} \right)$

where (p^h) is the probability that a cell is hit h times

where $(1-p)^{D-h}$ is the probability that a cell is missed $D-h$ times

and $\frac{D!}{h!(D-h)!}$ is the binomial coefficient (≥ 1) that accounts for all the possible combinations of hits and misses when D events are measured in the population.

Equation 2 is merely a probability equation for any cell being hit h times; it does not allow for the probability that a cell may survive after a hit is registered. It is therefore necessary to introduce a term to cover the probability that a cell may survive if it is hit h times:-

Equation 3 P (Net Survival Probability) $= (p^h)(1-p)^{D-h} \left(\frac{D!}{h!(D-h)!} \right) H(h)$

where $H(h)$ is the hit survival function that represents the

probability a cell can survive with h hits.

The net survival probability (equation 3) is therefore the product of the PROBABILITY that a cell is hit h times and the PROBABILITY that the cell can survive with h hits.

To determine the TOTAL SURVIVAL PROBABILITY i.e. the probability that a cell can survive 1,2,3 or more hits it is necessary to sum the individual survival probability for each of these 1,2,3 or more hits:-

$$\text{Equation 4 Total Survival Probability} = \sum_{h=0}^D (p)^h (1-p)^{D-h} \left(\frac{D!}{h!(D-h)!} \right) H(h)$$

where $\sum_{h=0}^D$ represents the summation of the survival probabilities of zero (h=0) hits to the maximum possible hits (D).

If a cell received no hits then its survival probability must be 1 i.e. $H(h) = 1$ when $h = 0$. If we introduce the assumption that a cell is inactivated (killed) when it receives one or more hits (i.e. one or more targets are hit) the probability of cell survival must be zero i.e. $H(h) = 0$ when $h \geq 1$. Thus when these assumptions are made the total survival probability (equation 4) reduces the probability of survival to the probability of receiving no hits i.e. for a cell to survive it must not receive any hits (h=0). By substituting zero for the hit survival function ($H(h)=0$) when $h \geq 1$ and by substituting one ($H(h)=1$) when a cell receives no hits the total survival probability (equation 4) is simplified viz:-

$$\begin{aligned}\text{For } h=1 \text{ total survival probability} &= (p^1)(1-p)^{D-1} \left\{ \frac{D!}{1!(D-1)!} \right\} \times 0 \\ &= 0\end{aligned}$$

$$\text{for } h=0 \text{ total survival probability} = (p^0)(1-p)^{D-0} \left\{ \frac{D!}{0!(D-0)!} \right\} \times 1$$

$$= 1 \times (1-p)^D \times 1 \times 1$$

$$= (1-p)^D$$

$$\begin{aligned}\text{and therefore the total survival probability} &= 0 + (1-p)^D \\ &= (1-p)^D\end{aligned}$$

Therefore the total survival probability for a cell surviving radiation dose D if one or more hits causes inactivation:-

$$\text{Equation 5 Total Survival Probability} = (1-p)^D$$

From the identity $(1-p)^D = e^{D \ln(1-p)}$ (equation 6) it is possible to rewrite equation 4 as:-

$$\text{Equation 7 Total survival probability} = e^{-P_o D}$$

where $P_o = -\ln(1-p)$ when p is the probability that a cell receives no hits.

$$\text{Equation 8 TOTAL SURVIVAL PROBABILITY} = e^{-\frac{D}{D_o}}$$

where D_o is the mean lethal dose per cell, i.e. the average dose absorbed by each cell before it is killed for cells inactivated after receiving one or more hits. This equation is an exponential relationship and demonstrates that a single hit to kill model can be expressed as an exponential survival curve.

Along similar, but more simplified, reasonings the "single

hit to kill" model can again be demonstrated as exponential. The probability that a target will receive an energy absorption, or hit, occurs randomly and is governed by the Poisson distribution (Alper, 1979). Thus when a population of cells has been exposed to irradiation of a dose D_0 that would produce, on average, a single hit for every cell in the population a fraction $1/e$ would have experienced no hits while $1-1/e$ would have experienced one or more hits. That is the fraction $1/e$ (0.37) will survive and the fraction $1-1/e$ will die. If a further dose D_0 is given then the fraction $1/e$ of the initial population of $1/e$ cells will survive giving a final surviving fraction of $(1/e)(1/e) = (1/e)^2 = 0.37 \times 0.37$. This can be extended infinitely for further doses. Therefore after a dose D the surviving fraction S is given by:-

Equation 8 $S = e^{\frac{-D}{D_0}}$

If the surviving fraction is plotted on a log scale as a function of dose the curve will be a straight line passing through zero dose at 100% activity. This is the exponential curve described by Wood (1953) for the haploid strain of yeast.

Both these explanations derive an exponential survival curve from the single hit to kill model for radiation-induced death which explains cell death as being due to the inactivation of the only target.

I.6 Sigmoid survival:- Mathematical approach

If a cell contains a number of targets, of which more than one must be hit to cause inactivation or cell death, a multitarget single hit model can be proposed that extends the single hit to kill

exponential model already described. For such a model it is necessary to make the following assumptions.

- 1) Each cell contains n targets;
- 2) Each of these n targets has an equal probability of being hit (q);
- 3) One hit will inactivate one target.

For a cell to survive an irradiation insult at least one of the targets MUST NOT be hit i.e. one target remains unaffected by the irradiation. The probability of any ONE target, after receiving an irradiation of dose D , not being hit is equivalent to equation 8 for the single hit to kill or exponential survival curve,

$$\text{i.e. } S = e^{-\frac{D}{D_0}}$$

where D_0 is the average dose required to produce a single hit for every target in the population. The probability, therefore, for the INACTIVATION of a SINGLE target (i.e. one being hit) =

$$1 - e^{-\frac{D}{D_0}}$$

As the cell will contain more than one target then the probability of an entire cell being inactivated is equal to the probability of ALL the targets being hit or inactivated:-

$$\text{Equation 9 } I = (1 - e^{-\frac{D}{D_0}})^n$$

where I = probability of cell death or ALL targets being inactivated.

where n = the total number of targets that have to be inactivated.

The probability of cell survival can therefore be expressed as:-

Equation 10 $S = 1 - (1 - e^{-\frac{D}{D_0}})^n$

where S is the probability of cell survival with n targets inactivated.

This survival curve can be expanded

$$S = 1 - (1^n - ne^{-\frac{D}{D_0}} + (e^{-\frac{D}{D_0}})^n)$$

$$= 1 - 1 + ne^{-\frac{D}{D_0}} - (e^{-\frac{D}{D_0}})^n$$

$$= ne^{-\frac{D}{D_0}} - (e^{-\frac{D}{D_0}})^n$$

When D becomes large (in relation to the other terms) the term $(e^{-\frac{D}{D_0}})^n$ will become very small and can be ignored. The equation can therefore be approximated to

Equation 11 $S = ne^{-\frac{D}{D_0}}$ for high values of D.

This equation, and therefore this portion of the curve is exponential when D is relatively large. When D is small the curve will not be exponential and consequently for a range of irradiation doses the curve will be identical to Fig I.1D i.e. at low doses there is a shoulder and at high doses the curve becomes exponential. This describes a sigmoid curve.

When equation 11 is transformed to:-

$$\ln S = \ln n - D/D_0$$

it can be seen that if the exponential portion of the curve is

extrapolated back to zero it will intersect the abscissa at the point n . This is termed the extrapolation number which, in general terms, represents the number of targets per cell. Exponential curves are therefore examples of single hit to kill models and sigmoid curves are multitarget single hit models.

I.7 Survival curve parameters

To compare the irradiation induced sensitivity of different cell types it is necessary to plot survival curves in order to measure the effect different radiation doses have on a cell's survival; the results are expressed on a semilogarithmic plot. Description of survival curves require several pieces of information including the values for D_0 and the extrapolation number.

I.7.1 Mean lethal dose (D_0)

As already discussed the theory behind radiological induced damage assumes a target theory or concept. The value for D_0 is described as the mean lethal dose or the minimum dose required to "inactivate" or "hit" every possible target in the population. The distribution of radiation induced events will occur randomly and the probability for a target to be hit is determined by the Poisson distribution (Alper, 1979). Subsequently the theoretical dose of D_0 which should hit every target once will hit some targets more than once and miss others altogether. Cells whose targets have been missed will survive the irradiation onslaught and, according to the Poisson distribution, the surviving cells will be represented by the fraction of $1/e$ ($1/e = 0.37$) of the initial population. Therefore the dose D_0 reduces the surviving cells to the fraction of 0.37 of the initial population (Fig I.2). In other words the dose D_0 will miss $1/e$ targets

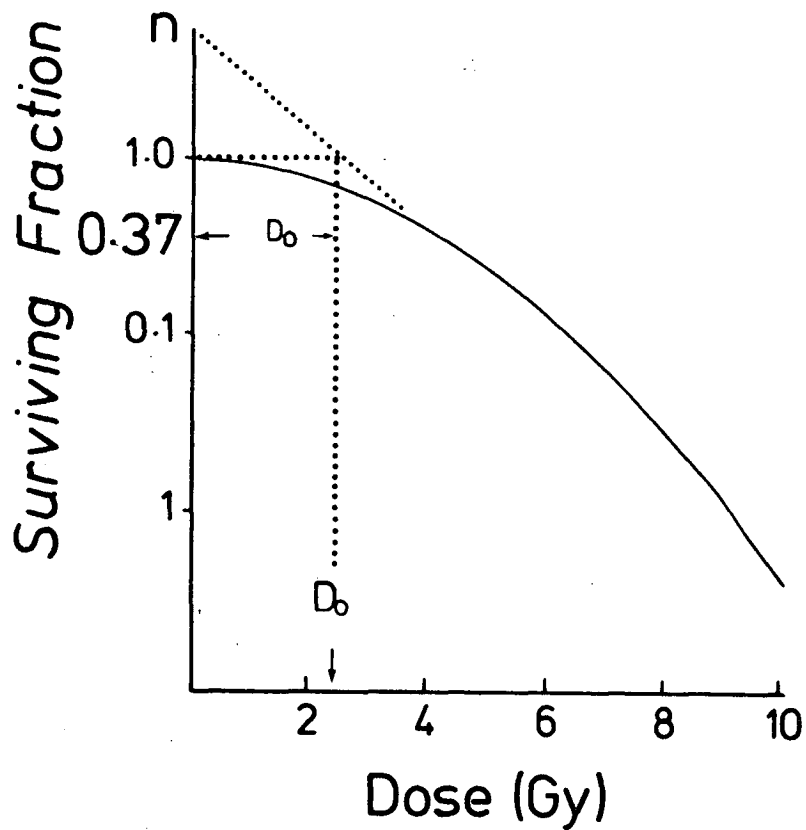


Fig I.2

Survival curve parameters

n extrapolation number. Y-axis intercept when the linear portion of the survival curve is extrapolated to zero irradiation dose

D_0 mean lethal dose. Irradiation dose that reduces the surviving fraction to 37% of the original value. This value is always calculated from the linear portion of the survival curve.

(37%) and allow this fraction to survive. This D_0 value is always calculated from the linear portion of the survival curve.

I.7.2 Extrapolation number (n)

The extrapolation number is the Y-axis intercept when the exponential portion of the survival curve is extrapolated back to zero dose (Fig I.2). The numerical value of n reflects the width of the nonlinear or shoulder region of the survival curve and represents the number of targets per cell that have to be inactivated to cause cell death. This concept is an oversimplification because other factors (e.g. repair mechanisms) can contribute to the extrapolation number. In general terms the extrapolation number is a numerical value that reflects an amount of sublethal damage that accumulates prior to cell death. This build up of sublethal damage could be attributed to the number of targets per cell as well as the cell's ability to repair sublethal damage.

I.8 Repair mechanisms

The radiation repair model proposes that during low irradiation exposures cells survive because they possess a mechanism that "repairs" irradiation induced damage. This explains the shoulder region of the survival curve. This repair mechanism is ineffective at high irradiation doses due to a "system overload" resulting in cell death and producing the exponential portion of the survival curve.

Elkind and Sutton (1959 & 1960) first suggested that cells could recover from a build-up of sublethal lesions. In their experiments they utilised the fact that mammalian cells, when exposed to irradiation, produce shouldered (i.e. sigmoid) survival curves. If a cell population was irradiated with a dose from the exponential

region of the shouldered survival curve (e.g. 5 Gy in Fig I.3) it would be reasonable to assume that the cells surviving will be sublethally damaged, but still viable. If these cells were immediately exposed to further irradiation the curve will continue in the exponential manner (dotted lines). If these cells were rested before they received further irradiation repair mechanisms would operate to enable recovery from the sublethal lesions. These repaired or recovered cells should essentially behave as the original, nonirradiated population i.e. in response to low irradiation doses sublethal damage must accumulate before cell death is registered; this accumulation would be expressed as the shoulder on a survival curve (solid line). By exposing the rested/recovered cells to more irradiation and superimposing the resulting survival curve with that of the continuously irradiated population it would be anticipated that the two curves would not be identical. The fractionated/irradiation protocol would express a second shoulder, shown in Fig I.3. This was in fact observed by Elkind and Sutton (1959 & 1960) and provides good evidence for repair mechanisms.

To elaborate on the concept of repair models it is necessary to incorporate the target theory. If, for a theoretical example, a cell contains 4 targets all of which must be inactivated to cause cell death, then low irradiation doses will not inactivate all targets in every cell. When the irradiation dose is increased a level will be reached where small increments would be sufficient to inactivate the final target (i.e. the fourth target out of four) and cell death will result. At this stage the survival curve becomes exponential. If irradiation is terminated during the exponential phase then the cells with four inactivated targets will be dead but the

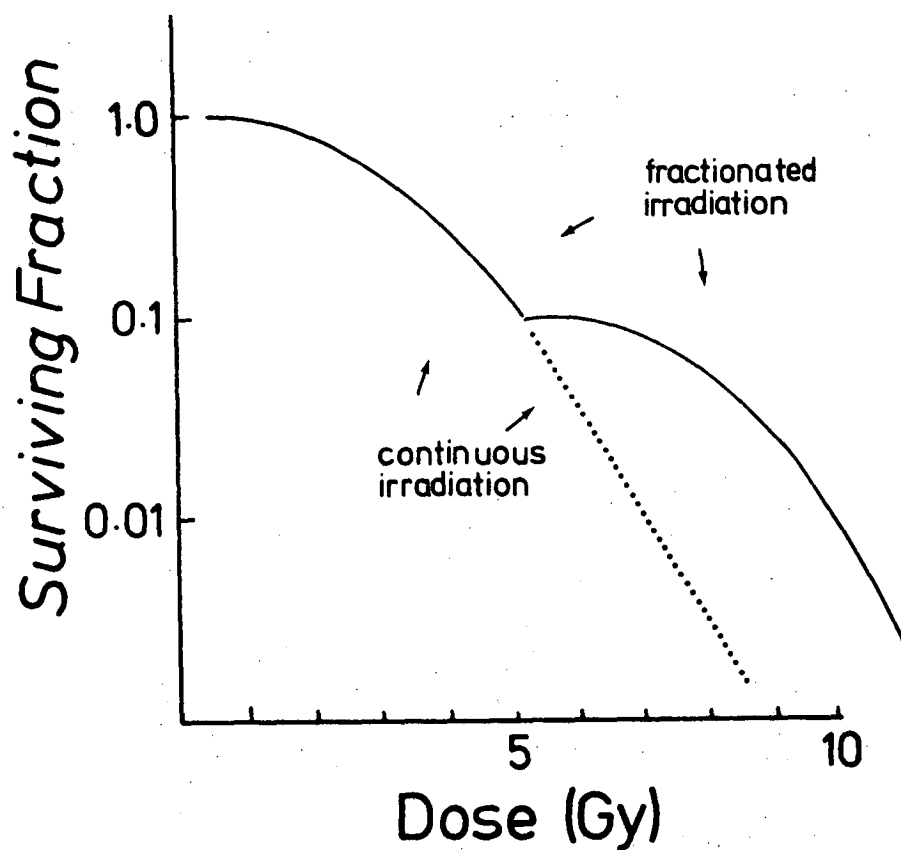


Fig I.3

Evidence for repair enzyme activity. Cells receiving continuous irradiation produced a survival curve with an initial shoulder and a linear portion. If the cells that received 5 Gy were rested before receiving further irradiation the surviving cells (i.e. those cells sublethally damaged) would recover. When these rested, recovered cells received further doses of radiation (fractionated irradiation) they would essentially behave as the normal, unirradiated population and, through their ability to withstand low doses of irradiation, produce a second shoulder. This has been termed Elkind recovery and has been described with a number of different cell types.

remaining cells with 3 or less inactivated targets (i.e. one or more targets unaffected) will be viable. It is within these cells that repair processes operate to restore the original quota of four targets. When these recovered cells are exposed to further irradiation doses all four of the recovered targets must again be inactivated to cause cell death; a shoulder will be expressed on the survival curve. This was demonstrated by Elkind and Sutton (1959 & 1960) and the recovery process was termed "Elkind recovery". Further such recoveries have been reported for a variety of cell types including algae (Jacobson, 1957), yeasts (Bacchetti and Mauro, 1965) and plant cells (Hall and Lajtha, 1963).

I.8

Summary

The aim of this appendix was to briefly review the area of radiobiology relevant to section 7. In order to quantitatively compare radiation sensitivities of different cell populations survival curves are analysed whereby the "surviving fraction" is plotted on a logarithmic Y-axis and the radiation dose is plotted on a linear X-axis. Surviving fraction designates a measured cellular function which is usually the cell's ability to divide as recognised by their colony forming capacity. There are two types of survival curves, exponential and sigmoidal; both of these curves can be mathematically described by regarding the cells as containing vital structures or "targets" whose integrity is essential for the cell's overall survival. An exponential survival curve results from the inactivation of only one target per cell. These cells can not accumulate sublethal damage and a single event is sufficient to cause lethality (Fig I.1B). A sigmoidal survival curve expresses a shoulder at low irradiation

doses and becomes exponential at higher doses and results from the inactivation of more than one target per cell and possible repair enzyme activities. These cells accumulate low doses of irradiation which is recognised on the survival curve as the shoulder at low irradiation doses (Fig I.1D). The larger the shoulder, which is reflected in the extrapolation number (n), the greater the degree of repair. The theoretical irradiation dose that could inactivate all targets within a population is designated as D_0 . Based on Poisson probabilities this dose will miss, and therefore allow the survival of, $1/e$ targets and therefore reduce survival to 0.37 or 37% of the initial population.

APPENDIX TWO

IRRADIATION DOSE RESPONSE CURVES

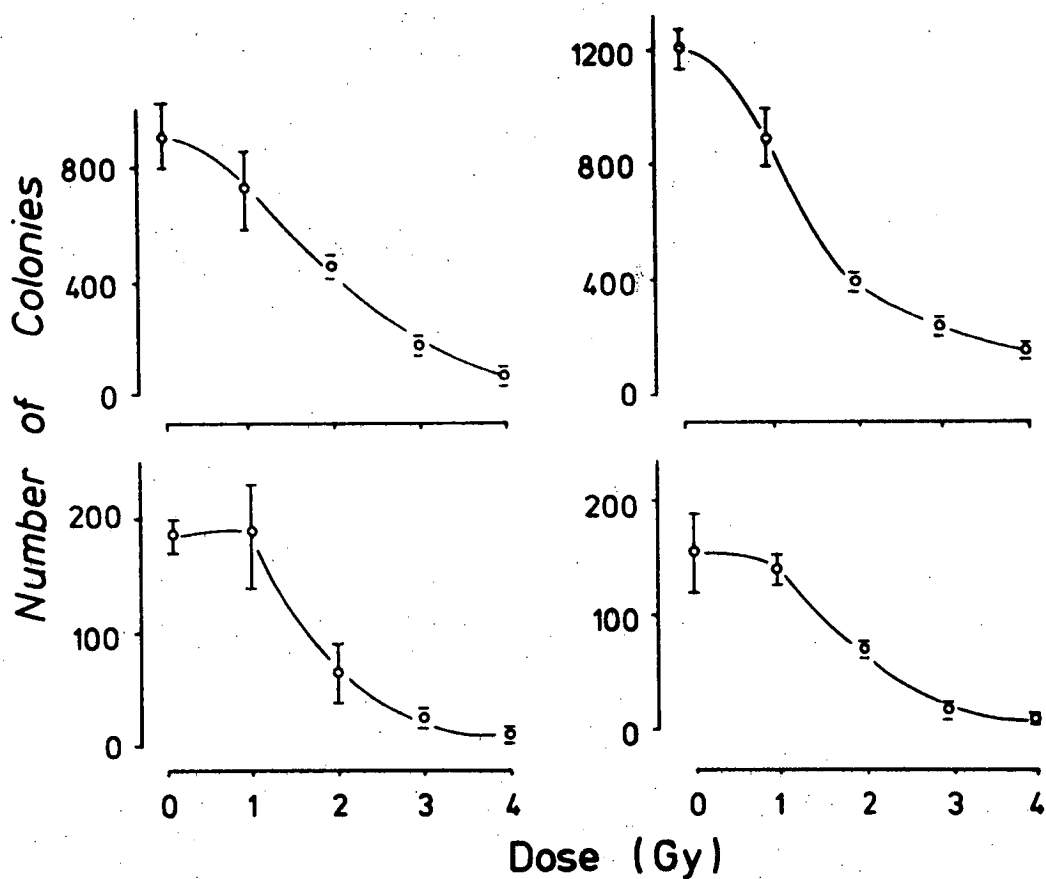


Fig II.1

Irradiation dose response curves for colony formation when cells were irradiated in liquid culture, incubated for 24 h before PHA was added and then incubated for a further 20 h in liquid phase prior to seeding into agar medium (scheme A of Fig 7.A). Results represent the mean \pm the standard deviation when triplicate plates were analysed from each of the four experiments. These values were converted into survival data and replotted as Fig 7.2A.

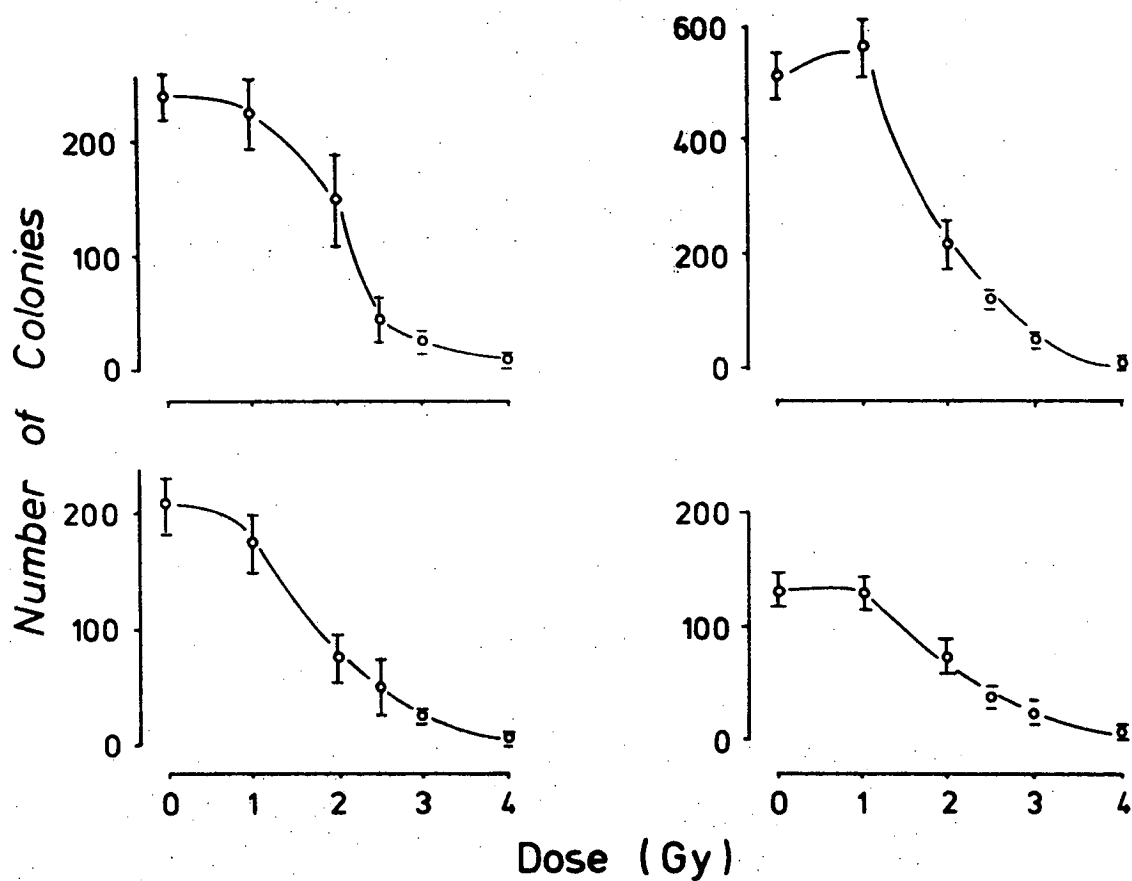


Fig II.2

Irradiation dose response curves for colony formation when cells were irradiated in liquid culture, PHA was immediately added and the cells were incubated for 20 h prior to seeding into agar medium (scheme B of Fig 7A). Results represent the mean \pm the standard deviation when triplicate plates were analysed from each of the four experiments. These values were converted into survival data and replotted as Fig 7.2B.

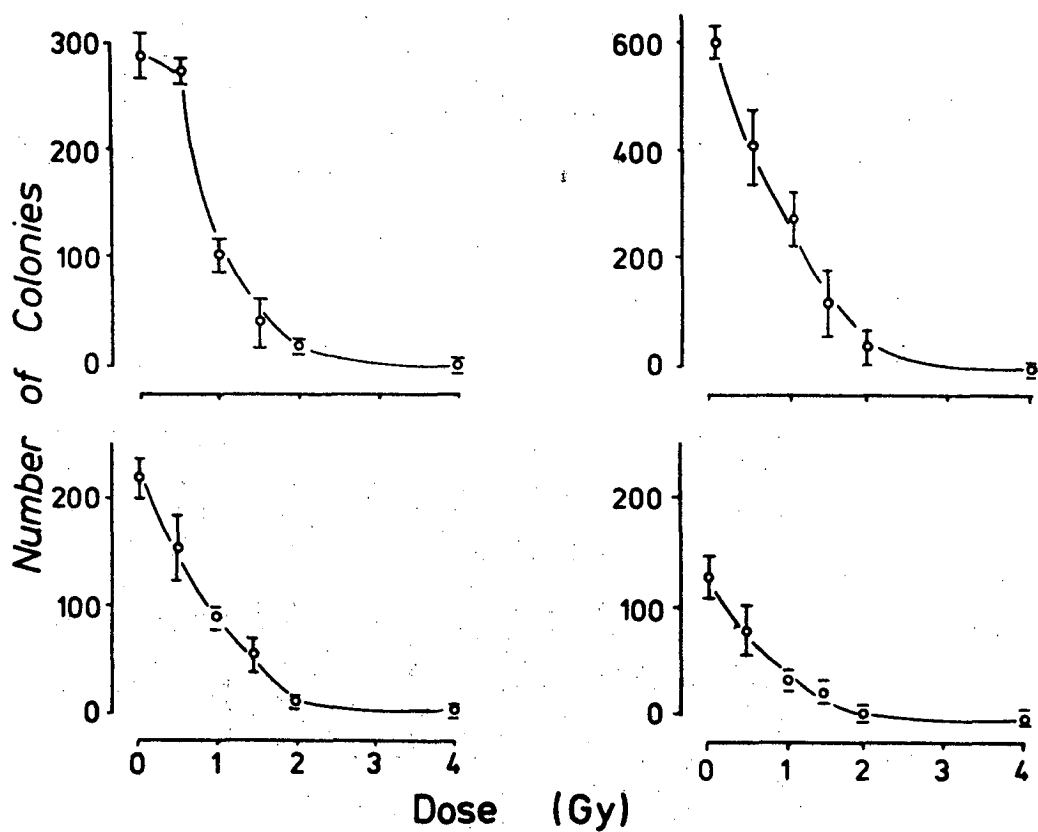


Fig II.3

Irradiation dose response curves for colony formation when cells were incubated with PHA for 20 h prior to seeding into agar medium. Immediately after the cells were seeded into agar they were irradiated (scheme C of Fig 7A). Results represent the mean \pm the standard deviation when triplicate plates were analysed from each of the four experiments. These values were converted into survival data and replotted as Fig 7.2C.

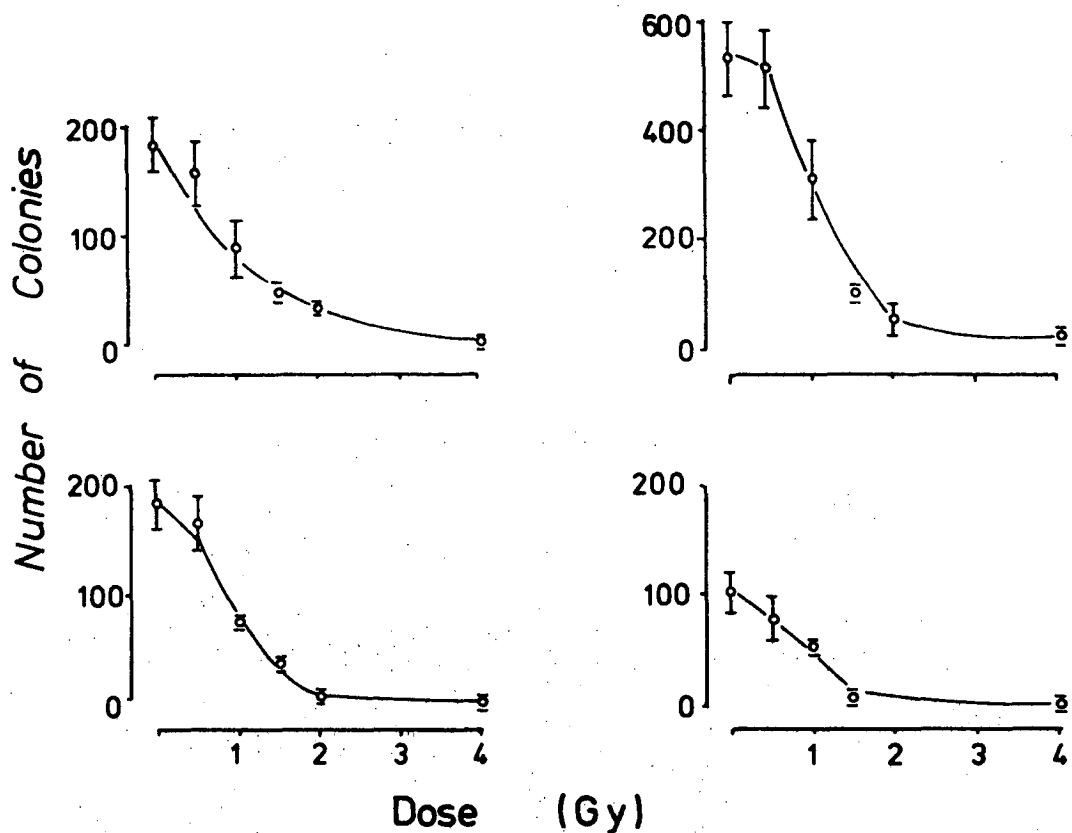


Fig II.4

Irradiation dose response curves for colony formation when cells were incubated with PHA for 20 h prior to seeding into agar medium. These agar cultures were incubated for 2 days before they were irradiated (scheme D of Fig 7A). Results represent the mean \pm the standard deviation when triplicate plates were analysed from each of the four experiments. These values were converted into survival data and replotted as Fig 7.2D.

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